

**THE RESPONSE OF
THE CILIATED EPITHELIUM
DURING AND AFTER EXPOSURE TO
IONIZING RADIATION**

A Physiological and Morphological Study

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Title and subtitle THE RESPONSE OF THE CILIATED EPITHELIUM DURING AND AFTER EXPOSURE TO IONIZING RADIATION. A Physiological and Morphological Study.			
Abstract Irradiation of the ciliated tissues of the body gives undesirable sideeffects. <u>In vitro</u> irradiation (10 Gy) of the rabbit's trachea shows that 1.5 Gy of indirectly ionizing radiation (50 kV and 6 MV X-ray, ⁶⁰Co-gamma 1.25 MeV) causes a 20 per cent increase of the ciliary beat frequency lasting 5-10 seconds, followed by a decline to normal ciliary activity during the ensuing course of irradiation. Electron radiation (4 MeV) proved to be three times more effective than photon radiation in regard to the physiological response of the cilia to ionizing radiation. This finding led to introduction of the concept "Relative Physiological Efficiency" (RPE) in this study, complementing the Relative Biological Efficiency (RBE) concept. This momentary increase in frequency can be caused by a radiation-induced increased hydrolysis of the ATP available in the cilia. The ciliary activity was 20 per cent lower than normal at 45 min following irradiation (10 Gy, 50 kV X-ray), whereupon it increased to 12 per cent above normal activity at two hours after initial irradiation. At re-irradiation (10 Gy, 50 kV X-ray) administered two hours after initial irradiation, the cilia showed a constant rate of activity. <u>In vivo</u> irradiation (10 Gy, 160 kV X-ray) of the trachea of the rabbit caused a heightened activity (10%) during the first three days after irradiation, indicating a <u>stimulation</u> of the ATP-synthesis. During days 4 to 8 after irradiation, the ciliary epithelium's morphology was <u>damaged</u> resulting in reduced transport ability. <u>Repair</u> took place during days 9 and 10 after irradiation, i.e. the function of the ciliary epithelium appeared to be restored. The membrane potential of the ciliary cell, registered <u>during</u> irradiation (10 Gy, 50 kV X-ray) showed no changes, which supports the assumption that the increased ciliary beat frequency recorded <u>during</u> irradiation can be due to rapid radiation-induced biochemical changes that are connected to the motility of the cilia.			
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THE RESPONSE OF THE CILIATED EPITHELIUM DURING
AND AFTER EXPOSURE TO IONIZING RADIATION
A physiological and morphological study.

BO BALDETORP

Civilingenjör, Ssk

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A Physiological and Morphological Study

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Sweden**

Lund 1984

*To Irma
Louise*

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I. Influence of temperature on the activity of ciliated cells during exposure to ionizing radiation.
Baldetorp L., Håkansson C.H. and Baldetorp B.
Acta Radiol. Ther. Phys. Biol. 16: 17-26, 1977.
- II. Intracellular recording of potential oscillations in ciliated cells exposed to ionizing radiation.
Baldetorp B., Baldetorp L. and Håkansson C.H.
Acta Radiol. Oncology 23: 49-53, 1984.
- III. A comparison between the immediate effects of photon and electron radiation on the ciliary activity of the rabbit's trachea. An in vitro study.
Baldetorp B.
Accepted for publication in Acta Radiol. Oncology 1984.
- IV. The effect of re-irradiation on the tracheal ciliary cell activity. A radiobiological study.
Baldetorp B. and Baldetorp L.
Submitted for publication.
- V. The effects of 10 Gy single-dose irradiation on the ciliated epithelium measured during and one-to-ten days following irradiation. A comparative physiological and morphological study.
Albertsson M., Baldetorp B., Håkansson C.H. and v Mecklenburg C.
Scanning Electron Microscopy 2: 813-824, 1984.

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PAPERS I - V

INTRODUCTION

The response of cilia to ionizing radiation is of great interest and importance in radiation therapy, as the distribution of the cilia is such that they must inevitably be irradiated during the treatment of tumours, whether they be situated in the brain, the respiratory system or in the genital tract. The side-effects of irradiation on the ciliated mucousal membranes may inhibit the patient's recovery - and it is therefore of utmost importance to clarify how radiation interferes with the normal activity of the cilia.

Experiments with cilia and irradiation were begun at an early date. The first to describe this interrelation was M. G. Bohn in Paris (1903). He had borrowed a small quantity of radium from Madame Curie and intended to carry out microscopic studies of the influence of radiation on the development of sea-urchin eggs. These eggs already have cilia in their blastula stage, and Bohn wrote: "Les mouvements ciliaires deviennent beaucoup plus intenses. . ." As a matter of fact, his finding was a stimulation of the ciliary activity that was presumably caused by the irradiation.

The stimulating effect of ionizing irradiation has since then been a matter of dispute. The acceleration of vital processes caused by irradiation is generally accepted and therefore "stimulation" would seem to be more a purely semantic question.

Radiobiologists have been searching for the true explanation of the effects after irradiation for decades, but experiments regarding the causes of events occurring in the cells during irradiation are conspicuously lacking in the research thus far conducted.

Baldetorp et coll. (1976) examined the beat frequency of the cilia from the rabbit's trachea during exposure to X-rays, and thus presented a new system of measuring the influence of the radiation on the physiology of cells. Not only was it now possible to detect early effects of irradiation, but even differences between various qualities of radiation could now be elucidated. This gave further information of value for common radiobiological concepts, e.g. that of the relative radiobiological effect (RBE), that

is based on the effects after irradiation.

Baldetorp and Håkansson (1977) found that the ciliary beating was stimulated during exposure to radiation, with a tendency to follow a particular pattern that is rather uniform at different dose rates.

The course of events found was difficult to explain, although simultaneous morphological studies were done. The hypothesis was presented that they were due to membrane-related changes influencing the ATP-concentration. Further investigation was called upon to elucidate unclear aspects of these events in connection with the immediate biological effects of irradiation.

EXPERIMENTAL GOAL

The field of radiobiology is so extensive that it ranges from the radiolysis of water - the immediate interaction between radiation and matter, the course of which is under 10^{-12} seconds - to the interaction of radiation with living tissue that has been investigated during many years after administered irradiation. These latter investigation are limited solely to analysing the effects after the dose has been delivered.

It is first during recent years that the events during irradiation have been investigated using a system where the activity of ciliated cells is studied. This research can be described as radiophysiological; physiology and morphology are, however, intimately interwoven, so that the present investigation has found it desirable to co-evaluate observations from both disciplines using modern ultrastructural methods.

The present investigation is a continuation of previously started work on the effects of irradiation on tracheal ciliated cells, and can be outlined in the following points:

- Examination of the influence of temperature on the early effects of radiation (I).
- Recording of the intracellular activity during irradiation (II).
- Comparison between the immediate effects of photon and electron radiation (III).
- Investigation of the biological reactions to two consecutive irradiations (IV).
- Correlation between physiology and morphology during and after irradiation (V).

THE MORPHOLOGY AND PHYSIOLOGY OF THE CILIA

There are chiefly two types of motile systems in eucaryotic cells which are dependent on the hydrolysis of adenosine triphosphate (ATP). That system which is represented in the muscle cells, among others, is made up of the protein actin and its mechanochemical connection with the ATP-ase myosin. The other system is based on the protein tubulin and its ATP-ase dynein. Ciliated cells are typical examples of those based on the latter system.

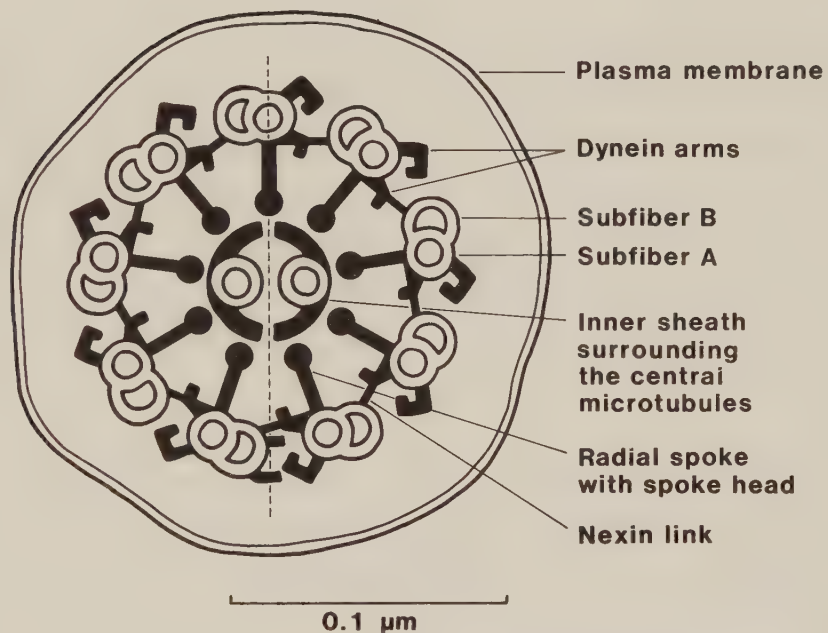


Figure 1. Cross-section of a cilium, viewed from the base to the tip. The dotted line defines the plane of the cilium's effective stroke. The outer doublets are designated clockwise with the numbers of 1 - 9 with respect to this line.

In order to demonstrate the structure and function of the cilia, a cross-section of a cilium as conceived today, seen from the above is shown in Figure 1. That part of the ciliary structure which lies within the ciliary membrane is called the axoneme and consists of a "9 + 2" pattern with nine outer doublets of microtubules and two single microtubules in the centre. Each outer doublet consists of a complete microtubule, subfiber A, and an imperfect subfiber B that is connected to it. Each subfiber A and the central pair of microtubules are made up of 13 protofilaments, while subfiber B consists of 10 - 11 protofilaments. The biochemistry and physiology of the microtubules have been described by Snyder and McIntosh (1976), among others.

The outer doublets are connected to each other by a protein called nexin (Stephens 1970, Linck 1973) . Two rows of arms (Afzelius 1959), dynein arms, extend from each subfiber A towards the subfiber B of the adjacent doublet. The outer doublets are numbered according to their position in relation to the central microtubules (Bradfield 1955, Afzelius 1959). Radial spokes extend from each subfiber A in towards the centre and have spoke heads on their ends that are associated with the central sheath, which in its turn surrounds the central pair of microtubules. The axoneme is surrounded by a double-structured plasma membrane which it has in common with the ciliary cell. The contents in a cilium is in direct contact with the cytoplasm of the cell via its basal body.

The cilia get energy for their movements from the hydrolysis of ATP (Gibbons 1965, Gibbons and Rowe 1965), which partly exists within the cilia themselves, and is partly synthesized from the abundantly occurring mitochondria in the apical part of the cell. It is believed that the ATP-ase activity is in the arms extending from subfiber A (Afzelius 1959, Gibbons 1963). The ATP-ase of the cilia is called dynein (Gibbons and Rowe 1965), and constitutes a part of these arms. Gibbons and Gibbons (1973) showed that dynein ATP-ase was involved in generating the energy for the strokes of the cilia, probably by some form of "cyclic cross-bridge mechanism" between the dynein arms and the microtubules. These arms interact with the doublet microtubules and generate ciliary beats by a sliding of adjacent doublets, substantiating the hypothesis of Afzelius (1959), which he based on experiments with sperm tails. This has later been verified by Satir (1965, 1968),

Summers and Gibbons (1971) and Warner and Satir (1974).

The ultrastructural similarity between the cilia from the lower organisms and the cilia from the mammalian trachea, makes it quite likely that their motility is conducted in the same way. The sliding mechanism is now thought to be a cyclic course of detachment and attachment of the dynein arms to the microtubules that is governed by the hydrolysis of ATP (Summers and Gibbons 1971, 1973, Sale and Satir 1977, Warner and Mitchell 1978, Takahashi and Tonomura 1978, Zanetti et coll. 1979, Mitchell and Warner 1980, 1981 and Satir et coll. 1981).

The tracheal cilia are about 7 - 8 μm long and have the diameter of 0.2 - 0.3 μm . Each ciliary cell has about 250 - 300 cilia on its surface (Sanderson and Sleight 1982). The ciliated epithelium consists of considerably more ciliated cells than the goblet cells. The cilia work in a layer of mucus that is a mixture of different kinds of secretions. Mucus is a visco-elastic, non-Newtonian liquid that consists of water, glucoproteins and other large protein complexes, to which oligo-saccharides are bound (Boat et coll. 1976). As early as 1934, Lucas and Douglas assumed that the mucus was divided in two layers, consisting of an upper mucousal layer covering a zone of mucus which is periciliary, serous and of low viscosity. This was later verified by Sadé et coll. (1970) and Sanderson and Sleight (1981).

When the cilia perform active strokes, their apices reach up to the upper mucosal layer and move it while the recovery strokes takes place with the cilia bent down in the serous mucus. An effective transport of the mucus takes place, as the cilia work together in a coordinated metachronal course of action (Sanderson and Sleight 1981). It is believed that the ciliary crown branching out from the ciliary apex, observed with transmission electron microscopy (TEM) by Dirksen and Satir (1972), and by Kuhn and Engleman (1978), can further assist in the transport of the mucus (Jeffrey and Reid 1977).

MATERIAL AND METHODS

This work is based on experiments performed on the trachea of the rabbit. A total of 194 healthy animals were used, weighing between 1.9 and 2.5 kg; they were thus full-grown and sexually mature. As no difference has ever been demonstrated between male and female animals as far as these experiments are concerned, the sexual distribution of the experimental material is not accounted for.

Tracheal specimens from 169 animals were irradiated with 10 Gy of photon energy and the tracheas from 10 other animals were irradiated with 5 Gy of electron energy, after which they were placed in an experimental chamber (I - IV). A further ten animals were irradiated (10 Gy) in vivo under anaesthesia (pentobarbital, 40 mg/kg) (V). In one of the investigations (II), five tracheas were used as controls.

When the trachea is removed from the animal's body, there is a sudden collapse of the preparation due to contraction of the connective tissue and the smooth muscles. The tracheal specimen must therefore be stretched to the length it had in the body of the animal prior to the excision. This manipulation causes physiological changes, so that an adaptation period in the experimental chamber was necessary. Conditions were stabilized within the 30 minutes chosen for this period, with quite good margins.

The studies consisted of recording the beat frequency of the cilia using a "light-beam" reflex method that has been common during the last twenty years. The ciliary surface of the trachea was illuminated with "cold light" and the ciliary movements were seen under the microscope as blinking reflexes. The microscope used for studying the preparations was equipped with a photodiode that transformed the light reflexes to electrical signals which were registered on the mingograph. This light beam reflex method enables registrations of the beat frequency while the tracheal preparation is in a horizontal position in the experimental chamber and is being irradiated with a vertical beam. The electrophysiological measurements performed with a glass capillary electrode mounted on a micromanipulator in accordance with accepted methods also permitted registration during the course of irradiation.

The ciliary beat frequency was measured each second in all of the preparations. The frequency was stable after the adaption process, and the average value of the measurements done 30 seconds immediately prior to irradiation in each animal was taken as a reference value. Each rabbit was thus its own control.

The results of these second-by-second measurements during irradiation were used for plotting the figures in all of the reports. The Department of Statistics at the University of Lund performed the statistical analysis of the curves.

Specimens of both irradiated and non-irradiated trachea were taken for both scanning electron microscopic (SEM) and transmission electron microscopic (TEM) studies. These specimens were taken immediately prior to and following dose administration. The procedure used in preparation of the tracheal samples was in accordance with accepted ultrastructural methods and is described in paper V. The studies were performed on modern instruments (Zeiss EM 10 and Zeiss Nanolab EM, and a Cambridge Stereoscan Mark II).

The animals irradiated in vivo were examined as follows: ten animals were irradiated under anaesthesia. On the following day, one of the animals was sacrificed and its trachea was placed in the experimental chamber, where the ciliary beats were registered and the morphological specimens were taken. This procedure was repeated during ten consecutive days.

Irradiation in vitro was performed using a Philips contact therapy unit in part of the material (I, II, IV and part of V) and in the other part linear accelerators (Siemens Mevatron 6 and Philips M.E.L. SL 75) for high-energy photon and electron radiation (III) were used. For in vivo irradiation, a conventional roentgen therapy unit with 160 kV acceleration was used (Siemens X-ray unit). As irradiation at different energies was used, it was not possible to avoid variations in the dose rate. The irradiative technique is described in detail in the respective reports.

Radiation Geometry: This has not been uniform in all of the experiments due to the radiation energy. The "build-up" effect of the radiations must be considered. For 50 kV roentgen radiation an absorption in the ciliary epi-

thelium is calculated to 100 per cent (the mucus layer = 150 μm), when the beam is directly aimed at the lumen of the trachea. In order to get the available build-up when ^{60}Co and 6 MV photons were used, irradiation was performed beneath the bottom of the chamber. The irradiation thus struck the outside of the trachea before it struck its inner surface (the ciliary cells were irradiated from below). The irradiation had in these cases passed the lower part of the experimental chamber, which consisted of a perspex vessel containing water maintained at desired temperature, and the upper part of cotton wad soaked in Ringer's solution, before it reached the trachea. Thus, there was no extra build-up phenomenon in the pathway of the beam. The path of the irradiative beam within the chamber was 88 mm.

In vivo irradiation with 160 kV radiation was performed as described in paper V. In this case, the irradiation had to pass through the fur of the animal, its skin and connective tissue before reaching the trachea.

Irradiation with 4 MeV electrons was carried out directly towards the lumen of the trachea.

Dosimetry: In order to control the different radiation conditions, dosimetry was performed with thermoluminescent dosimeters (TLD) for the different qualities of irradiation. At in vitro irradiation, the dosimeters were placed in corresponding positions at the sides of and under the trachea, as well as in its lumen. The dosimetry of the in vivo irradiations was performed with ionizing chambers (V). The percentage of error in the dosimetric measurements including those in the dosimeters placed in the radiation source was approximated to be about 3.5 per cent.

RESULTS

Influence of temperature on the activity of ciliated cells during exposure to ionizing radiation (I).

Irradiation of the trachea at different temperatures (ranging from 20°C to 40°C) showed a stimulation of the beat frequency at all levels. A dose-rate of 0.34 Gy/s was used, and in comparison with irradiations done afterwards with a lower dose-rate, the frequency pattern differs from that of these latter trials in that it follows a slowly heightened and successively reduced curve-like function not having any true "peak" value, as found in the experiments III, IV and V. The curves were the whole time above the reference level. It can be asserted that the integration of the surface covered by the function of this curves reflects the effective work of the cilia. It is therefore concluded that more work is done at 20°C than at 40°C.

To interpret this finding, it must be assumed that the cilia need ATP in order to perform their work; more ATP is required at higher beat frequencies. The irradiation ought to have contributed the same amount of energy to the working system at all of the four temperatures used. Why, then, do the cilia beat faster at lower temperatures? It is only possible to present certain hypothetical explanations: 1. The mucus is more viscoelastic at the lower temperature, and as it is known that an increased burdening of the cilia gives - perhaps via signals from the ciliary crown (Dirksen and Satir 1972)- impulses to increase the work of the cilia, this together with stimulation from the irradiation may cause the increased beat frequency. 2. A higher availability of ATP due to the triggering of enzymes is another possibility (Ohyama et coll. 1974). 3. An increased hydrolysis of ATP in the pool already existent, by the radiation-induced enzyme release is a further possible explanation (Bacq and Alexander 1961). These experiments touch the field of radiobiochemistry, and therefore trials with ATP measurements during irradiation must be performed.

Intracellular recording of potential oscillations in ciliated cells exposed to ionizing radiation (II).

Ciliated cells have a membrane potential of 30 to 40 mV, inside negative. This potential fluctuates with an amplitude of about 1 mV and a frequency lying some few Hz above the ciliary beat frequency. Many studies consider this

to be intimately connected to the mechanical activity of the cilia (Håkansson and Toremalm 1966, Yanaura et coll. 1979). In order to ascertain if it could be influenced by ionizing radiation, measurements of the intracellular potential oscillations were performed with glass capillary microelectrodes. The examinations were initiated prior to irradiation in order to get the reference value, and continued during the course of irradiation. No effect on the electrical activity could be demonstrated. The frequency was completely stable during the entire course of irradiation. The beat frequency, on the other hand, increased during this period in the usual manner.

The conclusion to be drawn from the results of this experiment must be that irradiation does not cause any effects via an eventual electrical initiation of the ciliary beats. It appears more likely that the irradiation directly affects the cilium itself. It must here be pointed out that the ciliary beat frequency has never during any of the experiments been higher than the frequency of the intracellular oscillations.

This is interpreted to imply that the cilia are caused to beat according to a constant biological impulse rate, they must work in surroundings that offer strong resistance. The cilia are thus possibly not able to respond to every impulse.

A comparison between the immediate effects of photon and electron radiation on the ciliary activity of the rabbit's trachea (III).

A distinct pattern in the ciliary response could be observed when the trachea was irradiated with 6 MV photons and 4 MeV electrons. A stimulation effect was found, as at earlier radiation trials. The curves had in principle a similar course: first, a momentary increase to 5 - 10 per cent above the normal value, then a relative rapid increase up to about 20 per cent, followed by a decrease of the frequency increase.

There was, however, a difference between the curves: the maximal response to stimulation from electron radiation occurred at an absorbed dose of 0.5 Gy, while it took place at 1.75 Gy during photon radiation.

There was also another difference between the curves: the declination phase during photon radiation was the whole time above the reference level,

while at electron radiation it fell below the reference level already at an absorbed dose of 1.5 Gy.

The results of the trials described above show that electron radiation can be asserted to be three times as effective as photon radiation.

The effect of re-irradiation on the tracheal ciliary cell activity (IV).

As it was thought that two irradiations with a certain interval of time between them might give information on the consumption and eventual restitution of energy to the ciliary beats, two irradiations were performed on the same trachea with 10 Gy administered initially and after two hours had elapsed. Another trachea served as a control; it was stored two hours, after which it received initial irradiation. In each specimen where 10 Gy was given initially, the irradiation caused the same characteristic curve of stimulation (Figure in IV). None of the specimens given two irradiations showed the well-known "peak" response at the second irradiation.

As the beat frequency was continuously recorded in the two preparations, this lead to the discovery that when irradiation was stopped in those specimens given initial irradiation, there was a decrease of the ciliary beat frequency - greatest after 45 minutes following irradiation - by 20 per cent (Table in IV). The course of the curve then changed and there was an ensuing overshoot which reached 12 per cent immediately prior to the next irradiation. The pattern of the curve during the second irradiation indicated a reduction of the ciliary activity. At its lowest point this was 3.8 per cent above the normal level at an absorbed dose of 2.25 Gy, and towards the end of the irradiation period it slowly increased to 8.8 per cent, which was significantly above the reference level.

The effects of 10 Gy single-dose irradiation on the ciliated epithelium measured during and one-to-ten days following irradiation (V).

This paper accounts for a comparative physiological and morphological study. The ciliary beat frequency was measured during roentgen radiation (50 kV) and ^{60}Co radiation, and showed a rapid increase up to 22 - 25 per cent above the reference value after an absorbed dose of 1.3 - 1.5 Gy, followed by a gradual decrease during the remaining parts of these exposures (Figures 1 and 2, V).

The ciliary beat frequency of the area of the trachea irradiated in vivo was measured during ten consecutive days after the exposure and was related to the activity of a non-irradiated part of the same trachea. The beat frequency quotient was shown to be higher than 1.0 during at least three days after the irradiation. This was called the "stimulation" phase. During the following days, however, only parts of the mucous membrane exhibited ciliary motion, and the quotient decreased in the active areas to about 1.0 during days 4 - 8 (Figure 3, V). India ink applied to the mucous surface indicated a non-effective transport function during these days. This was regarded as a phase of radiation-induced "damage". During days 9 and 10 following irradiation, the mucociliary activity increased again, however, and the beat frequency in the irradiated part of the trachea showed the same values as those of the non-irradiated parts. The conclusion must be drawn that there was a "repair"-mechanism present.

Already 24 hours after irradiation, abnormal structural changes were observed in the form of blebs on the cilia, which could be seen both in the SEM- and the TEM-micrographs. These changes became even more apparent during the following days, and SEM studies showed a clear tendency toward progressive damage that was most pronounced during days 4 - 8 after irradiation. The specimens showed less and deleterious effects during the ensuing days, however, and on day 10 after exposure the tracheal cilia appeared to be completely restored.

In order to get a more certain estimate of the extent of this damage in relation to time, a scoring of the SEM-micrographs was done as to the deviations from normal appearance found on them. This scoring was done by four persons quite independently of one another. The average value of the scores given by these four persons was then plotted (Figure 9, V), the plotted graph showed a pattern that indicated a heavy process of damage around days 4 and 5 after irradiation. The concepts of "stimulation", "damage" and "repair" in their physiological implications were then included in Figure 9 (V), and their agreement was found to be mainly acceptable.

The diameter of the cilia was measured both on the TEM- and the SEM-micrographs. The value measured on the TEM-micrographs of a normal trachea was about 0.23 μm , which is the correct value, and it increased to 0.25 μm

on day 1 after irradiation. The diameter was $0.19\text{ }\mu\text{m}$ on day 4, and on day 10 the cilia again showed their normal diameter of $0.23\text{ }\mu\text{m}$. Statistical analysis showed that the differences were significant ($p < 0.002$).

The results found confirmed the description of the morphological process as it was described above.

DISCUSSION

All radiobiological effects on living objects are primarily to be found in the effect radiation has when it is absorbed in a cell or in a cellular system. It is, however, not certain that an immediate effect can be clearly measured. The heart does not change its frequency when the chest is submitted to radiation treatment; the EEG shows no change in the frequency pattern when the brain is irradiated. Radiation-induced changes can be detected first after a certain lapse of time (Garcia et coll. 1963, Garwicz et coll. 1975, Håkansson et coll. 1969). As the clinically interesting effects are naturally those to be observed after irradiation, there have only been a few reports published dealing with the effects on cellular systems during radiation treatment.

The ciliary cells are, from a radiobiological standpoint, a unique testing system, permitting studies with physiological methodics (i.e. the beat frequency), with electrophysiological methodics (intracellular potential measurements) and with biochemical methodics (ATP-measurements) at the same time as the ciliary epithelium is being irradiated. Ciliary cells are found in many parts of the body, and basic radiobiological research in this field therefore has clinical relevance, as it is nearly impossible to avoid the irradiation of cilia during the course of a radiation therapy.

Figure 2 illustrates how the physiological activity of the cilia (in the rabbit's trachea) in respect to different parameters of radiation physics has thus far been charted. Electron-microscopic studies have also been conducted in all of the trials in order to permit the detection of eventual changes occurring parallel to the physiological response.

It was found in all of the experiments that ionizing radiation stimulates the ciliary beat frequency, though not at re-irradiation. This phenomenon is no new discovery, having been described as early as 1903 by M. G. Bohn. It is, however, the charting of the morphology of the cilia (Afzelius 1959, 1961 a, b, Bradfield 1955, Dirksen and Satir 1972, Fawcett and Porter 1954, Gibbons 1960, 1961 a, b, Gibbons and Grimstone 1960, Kuhn and Engleman 1978, Manton 1952, Satir 1963, Watson and Hopkins 1962) and of their physiology (Dalhamn 1955, Frenckner and Richtner 1939, Goldhager and Back

1941, Håkansson and Toremalm 1965, 1966 a, b, 1967, 1968, 1971, Krueger and Smith 1957, Lucas and Douglas 1934, Mercke 1975, Proetz 1932, Reimer and Toremalm 1978) done during recent years that has enabled meaningful studies of the early radiobiological effects on the cellular level to be conducted.

The interpretation of the results is based on the assumption that the energy available to the cilia comes from adenosine triphosphate (ATP) (Gibbons 1965, Gibbons and Rowe 1965), and that the supply of ATP governs the ciliary beat frequency (Hoffman-Berling 1955, Brokaw 1961, 1975, Satir 1974).

It is of great interest for the entire field of radiation therapy to have knowledge of the effects of temperature, especially since hyperthermia has been introduced in the treatment of tumours. The investigations reported are in nearly all cases based on measurements at different temperatures after the administration of radiation. The results of these trials show that radiation lesions are greater when higher temperatures are applied on a tissue (Barth and Wachsmann 1948, Belli and Bonte 1963, Lindholm et coll. 1982, Martin and Caldwell 1922, Overgaard and Overgaard 1974). Hypothermic trials have mainly been conducted on cellular systems (Simpson 1916, Myers et coll. 1962, Myers and Sutherland 1962, Ohyama et coll. 1974). Early investigators have mainly found that low temperatures tend to promote cellular survival. It should be pointed out that the results treated here are from studies of the course also of events after irradiation.

Ohyama et coll. (1974) presented the hypothesis that the initial effect at 37° C stimulated phosphofructokinase, resulting in an increased degradation of ATP, but the reaction of phosphofructokinase was considerably less at 25° C, so that the cells had a normal supply of ATP and maintained their viability longer. "The initial lesion" discussed in their report is difficult to define, and this complicates applying their results to the present series of trials in order to see if an increased supply of ATP can occur at the lower temperature. An increased amount of ATP is possible at the lower temperature. Another question is whether or not ATP is equally susceptible to hydrolysis as it is at 37° C. The surface that can be calculated from the curves using Simpson's formula (Figure 1, I) shows beyond doubt that the cilia perform more work at the lower temperature.

Other factors must also be considered. It is well-known that the ciliary beat frequency increases when the ciliary cells are subjected to increased mechanical loading. At lower temperatures (20° C.) without irradiation there is a reduced ciliary beat frequency (Mercke et coll. 1974) This is not thought to be solely due to the usual reduction of physiological activity when temperature is reduced, but is also due to the fact that the cilia are beating in a viscoelastic medium, which further slows down the beat frequency at low temperature. This condition during irradiation is more difficult to comprehend. It must here be assumed that stimulation occurs. It can possibly reduce the mucous tenacity, the viscoelasticity of which is difficult to measure, as the layer of mucus covering the cilia is not a Newtonian liquid; the cilia can beat faster, but the effect can also be a direct effect on "sliding mechanism". Another possible reason can be the membrane effect on the mitochondria where the ATP-synthesis occurs (Baldetorp et coll. 1976). Further research is called for to shed light on this complex mechanism.

In an attempt to elucidate whether or not ionizing radiation causes changes in the electrical conditions of the ciliated cells, which eventually could contribute to an explanation of the increased beat frequency seen during irradiation, the membrane potential of the ciliated cells was examined simultaneously with the irradiation treatment (II).

The membrane potential of the rabbit's tracheal ciliated cell has previously been shown to be approximately -40 mV, and with a periodically fluctuating potential of 1 mV (Håkansson and Toremalm 1966 b). These earlier reports proposed an inter-connection between the oscillating signal, i.e. the so-called "pace maker" signal, and the ciliary beating, which has also been concluded by Yanura et coll. (1979).

The frequency of the potential oscillation was shown to be unaffected during the whole exposure of 10 Gy (Figure 2, II). The same pattern of the frequency change of the voltage oscillations was noted as in the untreated controls. This finding is not in agreement with the change in the beat frequency measured under the same conditions. The result presented may indicate that the ionizing irradiation interacts with other mechanisms involved in the ciliary motility machinery e.g. production of ATP in the mitochondria and diffusion of ATP from the cell up to the axoneme of the cilia.

These two mechanisms together with the biochemical activity involved in the ciliary motility determine the frequency of the ciliary beating (Brokaw 1966, Hoffman-Berling 1955). If the ionizing radiation thus stimulates one or all of these mechanisms, the beat frequency may increase.

The electrical conditions of the ciliated cells during irradiation were unchanged as far as the membrane potential was concerned. This is consistent with the findings of Bergeder (1958 a, b) and of Nachmannsohn (1957). They showed that high doses of ionizing radiation had to be administered to muscle and nerve cells before changes in their electrophysiology were detectable.

Previous investigations by Baldetorp and Håkansson (1977) showed that the beat frequency increase of the ciliated cells occurred nearly independently of the accumulated dose rates of photon irradiation 50 kV, 0.15 Gy/s and 0.05 Gy/s. In all of these experiments an absorbed dose of about 1.5 Gy was required before the maximal beat frequency appeared. To see if this biologic effect was influenced by the radiation energy, ciliated cells were irradiated with 6 MV photons (III). The dose rate was 0.07 Gy/s. This irradiation caused the same pattern of the ciliary reaction during the exposure as seen at low-energy irradiation.

From a physical point of view, the interaction between the radiation and the materia is not the same if 50 kV is compared to 6 MV. Photons below 20 keV interact mainly through photoabsorption, while high-energy photons interact via Compton effect and the pair-production process. The difference in energy did not see to be of any importance for the ciliary reactivity.

When the effects of 6 MV photons and 4 MeV electrons were compared (III), the physiological response of electrons was found to be greater than that of photons. 4 MeV electrons caused a maximal increase at 0.5 Gy, as compared to 1.5 - 1.75 Gy for photons. The decline of the electrons was also more rapid. This is not consistent with the common RBE-value. Therefore a new concept was introduced, the RPE (=relative physiological effeciency), and during the actual experimental conditions the value was stated for electrons/photons=3/1.

Cells exposed to 10 Gy photon irradiation showed weakened ciliary

activity first after the treatment (IV). Such decline has also been observed by Fujiwara et coll. (1972), and was thought to be caused by disturbances of the ATP-production. Reduced ATP-production following irradiation has also been reported by Skog et coll. (1983) after the irradiation of Erlich ascites cells. The frequency variations after irradiation in relation to the reference level are also shown in the Figure (IV). It also appeared desirable to test a second irradiation in both the decline and the overshooting phases. The last period was first re-irradiated. No initial frequency increase was seen during this exposure, which was made two hours following initial irradiation. Tracheas kept as controls two hours showed the usual pattern of a 20 per cent frequency increase at initial irradiation.

The overshooting that occurs later on has not, as far as is known, been earlier described for photon radiation. Ogi (1959), however, described a similar phenomenon for electron radiation. This can be understood as an initiation of a resynthesis of ATP, but not to such a degree that a "peak increase" occurs at re-irradiation. These studies ought to be complemented in future with re-irradiation at the lowest period of the declination phase.

Electron microscopical studies during these periods of 10 Gy irradiation did not show any certain evidence of either stimulation or damage. Such evidence was, however, obtained in the trials where the rabbits were irradiated in vivo and examined 1 - 10 days after treatment (V). It was there possible to follow stimulatory effects in the form of multilobulated nuclei, visible during the first days following irradiation in the TEM-micrographs, and also to study signs of damage during the following days, which were most clearly seen in the SEM-micrographs, where degradation was greatest on days 4 - 8 after irradiation. Signs of repair were also noted during days 9 to 10. Four persons evaluated the micrographs, independently of one another, using a scoring system suitable for graphic presentation (Figure 9, V). The morphology was then compared with the results obtained from the measurements of the beat frequency. In this connection it is also seen that the changes in the ciliary beat frequency that occurred during the first two hours following irradiation (IV), where it was reduced by 20 per cent, were later amended by a rise to about 12 per cent above the reference level. It is possible that this rise reflects a resynthesis of the ATP having long duration, as a beat frequency of about 10 per cent above the reference level is recorded during

the first three days following in vivo irradiation. Surfaces here and there were completely inactivated during the damage phase. A few other areas were intact but had reduced their beating frequency.

Radiophysical aspects

One of the most important concepts in the field of ionizing radiation is that of linear-energy-transfer (LET) (Zirkle 1954), which signifies the amount of energy per unit of length that the radiation beam deposits along its path. For electron radiation, which is directly ionizing, LET implies the amount of energy per unit of length deposited by the individual electrons, while for photon radiation LET indicates the value of the energy depositions made by the electrons that have been excited by the entering photons. LET is determined by the energy and the charge of the particles. The greater the charge and the lower the energy, the higher will LET be.

As the energy of roentgen radiation constitutes a spectrum, LET can vary over a large area, which implies that even a LET-concept using median values can be difficult to apply for comparing different irradiations with each other. The present investigation has used 50 kV and 160 kV roentgen radiation, ⁶⁰Co-gamma radiation (1.25 MeV), 6 MV photon radiation and 4 MeV electrons. The LET-values for the indirectly ionizing forms of radiation, as calculated on the basis of their track-average and absorbed dose-average, show much variance (ICRU Report No. 16, 1970). Despite this circumstance, the ciliary cells have an identical pattern of reaction during irradiation with the different qualities of indirectly ionizing radiation.

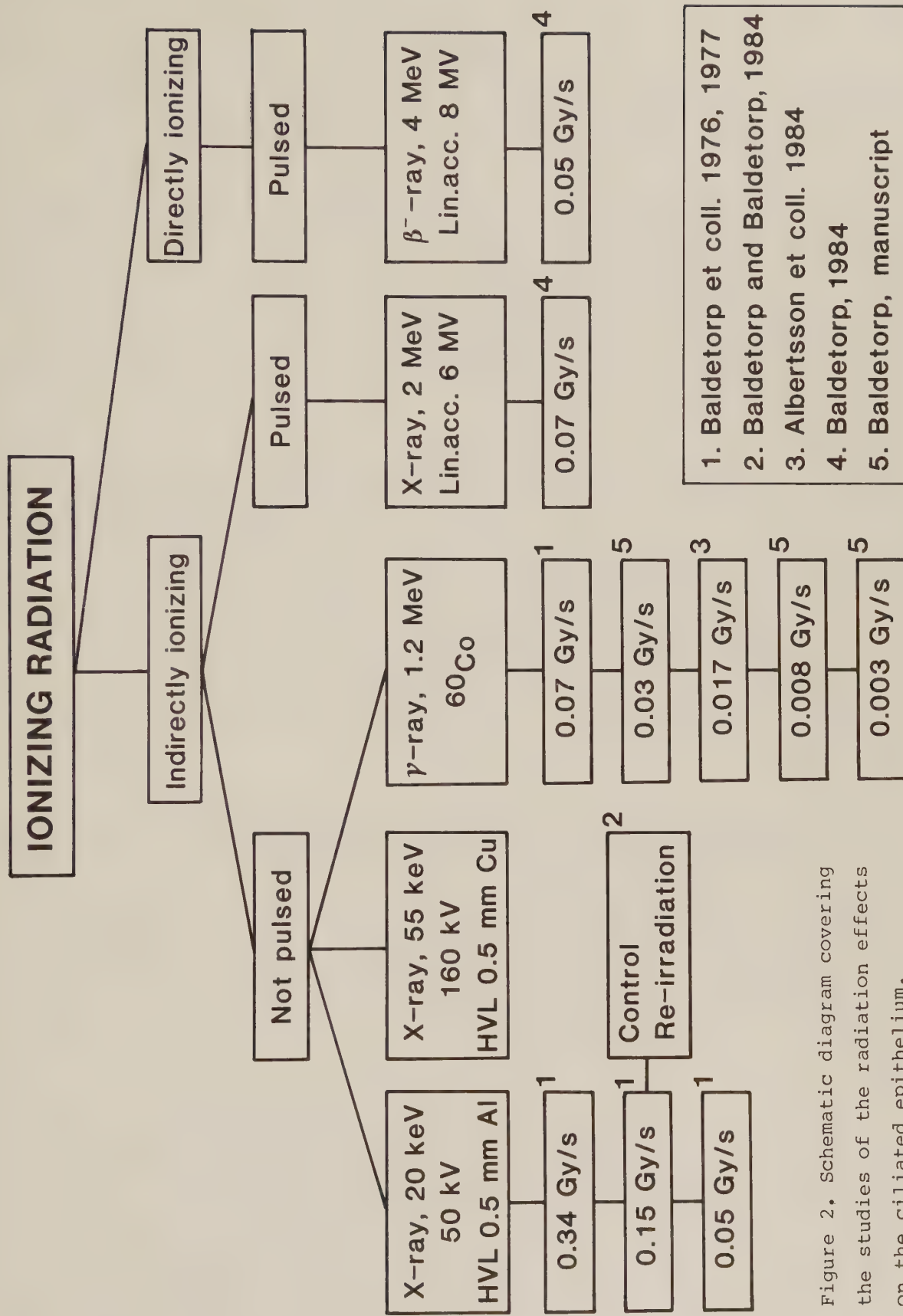
The 4 MeV electron radiation, which has the same LET-values as 6 MV photons, proved to be three times more effective for delivering the absorbed dose necessary to produce the maximal increase in the ciliary beat frequency. In radiation with higher LET, e.g. low-energy roentgen radiation, the ionizations take place very near each other. This causes a very high concentration of the free radicals H[•] and OH[•], but it is nonetheless not possible to get a higher degree of physiological effect using the higher LET at 50 kV roentgen radiation than is presented in the figure (V). A lower LET, e.g. 6 MV photon radiation, causes exactly the same ciliary response. According to HALL (1978), different radiations with the same LET can have different biological effects, so that electron radiation must have a special effect on

the physiological activity of the ciliary cells, a fact that was seen in project (III) of this investigation.

The radiation from roentgen tubes and radionuclides is continuous as regards time, which is not always the case with high-energy electromagnetic and particle radiation generated in accelerators. By use of advanced electronic control-systems, the radiation is directed out in the form of "radiation packages" having an extremely high dose-rate and a certain durability. The desired rate of administered dose-per-second is decisive for how many "radiation packages" are to be gated per second.

The present study (III) has used 6 MV photon radiation and 4 MeV electron radiation generated in linear accelerators which release the radiation in a pulse-wise manner. The length of this pulse for the radiation qualities used in project (III) was 5 microseconds for both of the accelerators. The pulse frequency is 150 Hz. This gives an average dose rate of 0.07 and 0.05 Gy/s in trachea, respectively. The fact that photon radiation is distributed periodically does not change the ciliary response as compared to the response seen during continuous radiations. The pattern of reaction shown by ciliary cells is namely nearly identical for e.g. ^{60}Co and for 50 kV roentgen radiation (Figure 1 and 2, V). In these two latter cases, it is the absorbed dose of 1.5 Gy that stimulates the cilia to beat faster, despite the fact that the radiation qualities have different energies. The clear difference in the reaction of the cilia seen at electron radiation can thus not be explained by the radiation being distributed in pulsed form.

The difference can be due to the differing microdosimetric attributes of the types of radiation used, and possibly be due to release of the electricity carried by electron radiation. In this latter case, there may be some comparison with the response of excitable cells to electric current. It can possibly excite the ciliary membrane with increased potential fluctuation as a result, and thereby increase the beat frequency. It therefore appears necessary to carry out electrophysiological studies at electron radiation.



1. Baldetorp et coll. 1976, 1977
2. Baldetorp and Baldetorp, 1984
3. Albertsson et coll. 1984
4. Baldetorp, 1984
5. Baldetorp, manuscript

Figure 2. Schematic diagram covering the studies of the radiation effects on the ciliated epithelium.

SUMMARY

These studies on the effects of ionizing radiation on the ciliated epithelium of the rabbit's trachea have led to the following results:

The effect of temperature on cilia exposed to 50 kV roentgen radiation:

A decided stimulation of the beat frequency was seen during the entire course of irradiation at the temperatures investigated, i.e. at 20° C, 30° C, 37° C and 40° C. This effect was found to be greatest at 20° C. This finding raises the question of an eventual inactivation of phosphofructokinase at lower temperatures, or possibly of effects from other enzymes.

Intracellular potential activity compared with the beat frequency of the ciliary cells during irradiation:

Electrophysiological measurements of the fluctuations of the membrane potential were made during the irradiation of the ciliary cells. No significant changes in the electrical activity were observed. As the ciliary beat frequency was nonetheless stimulated at the dose-rate administered (0.15 Gy/s with 50 kV roentgen), it is concluded that a radiation dose of 10 Gy does not affect an eventual relation of potential fluctuation to the physiological effect.

Comparison of 4 MeV electrons with 6 MV photons during administration of radiation:

Both qualities of radiation (10 Gy) stimulate the ciliary beat frequency, but the effectivity of electron radiation was calculated as being three times greater than that of photon radiation. This led to the introduction of the concept "Relative Physiological Efficiency" (RPE), scored as 3 in this experiment.

Effect of re-irradiation on the ciliary beat frequency:

An initial irradiation (10 Gy) gave the usual stimulation pattern of a 20 per cent increase in the beat frequency. This had, however, been reduced to 20 per cent under the normal value at 45 minutes following irradiation, but then rose to about 12 per cent above the normal level at two hours after completion of irradiation. Re-irradiation with 10 Gy at that stage did not stimulate the beat frequency. Another tracheal sample that had been stored

in the experimental chamber two hours was given an initial irradiation (10 Gy) with the usual increase in the beat frequency as a result. If the stimulation during initial irradiation can be considered as an increased utilization of ATP, then the following decrease in the beat frequency must be attributed to a reduced supply of ATP, the later restitution of which causes the frequency to again rise above the normal level. The finding that re-irradiation has no stimulatory effect is interpreted as meaning that the cilia are already working at their maximal capacity after having received the additional new production of ATP.

In vivo irradiation and subsequent in vitro studies of the beat frequency and ultrastructure:

The upper part of the rabbit's trachea was irradiated with 160 kV roentgen (10 Gy) after the animal had been anaesthetized. The lower part of the trachea served as a control. Ten rabbits were irradiated, and on each of the following ten days the trachea of one animal was investigated. The irradiated part of the specimen showed an increased beat frequency during the first three days following irradiation, in comparison with the non-irradiated part. During days 4 - 8 there was reduced mucociliary activity that agreed well with the ultrastructural signs of damage observed. The tracheal mucus was restored to its normal state days 9 - 10. There were three distinct physiological and morphological phases reflecting the course of events occurring in the beat frequency and the ultrastructure: a stimulation phase, a damage phase and a repair phase, all taking place during the ten days ensuing after an irradiation with a single dose of 10 Gy.

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INFLUENCE OF TEMPERATURE ON THE ACTIVITY OF CILATED CELLS DURING EXPOSURE TO IONIZING RADIATION

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Temperature has long been regarded as a factor capable of influencing radiation sensitivity, both in negative and positive ways. As far back as 1906 HART claimed that heating tissues made them hypersensitive to roentgen radiation, and that he obtained better results in neoplasm of the uterus, after hot intrauterine douches for several hours. On the other hand, SIMPSON (1916) regarded cold as a sensitizer for irradiation. He placed ice bags over the thyroid region in patients being irradiated for exophthalmic goitre and observed a more severe dermatitis in these patients than in those without ice bags. SIMPSON's opinion was shared by MARTIN & CALDWELL (1922), whereas HAWKINS & CLARK (1925) assumed that hyperthermia was a sensitizer for radiation. They made skin tests on guinea pigs, but kept the temperature rather high during the irradiation; therefore their findings of aggravated dermatitis following irradiation cannot be regarded as conclusive. Other authors also seem to assume that hyperthermia is a sensitizer for radiation and that a more intense effect on the skin was obtained if the irradiation was combined with heat (HALBERSTÄDTER & SIMONS 1923, BARTH & WACHSMANN 1948, STEWART 1976). EVANS (1941) found less reaction of the skin after irradiation at low temperature (0°C) than at room temperature. The influence of the temperature on the effect of irradiation of neoplastic tissue was investigated by MEYER & MUTSCHELLER (1937), and in experimental

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tumour-model systems by, among others, CRILE (1963), OVERGAARD & OVERGAARD (1972) and HAHN *et coll.* (1974). A significant increase of the biologic effect of the ionizing irradiation was found. A higher sensitivity of cells in tissue cultures irradiated at higher temperatures was reported by BELLI & BONTE (1963), CHAMBERLAIN & NORMAN (1966), HALL *et coll.* (1969), BEN-HUR *et coll.* (1974), KIM *et coll.* (1975) and LOSHEK *et coll.* (1976). OVERGAARD & OVERGAARD (1974) and ROBINSON & WIZENBERG (1974) were of the opinion that the heating time of the cells, in relation to the irradiation, was of no importance for the radiation effects.

The purpose of the present investigation was to attempt to analyse the influence of temperature on the early effects of ionizing radiation, to evaluate the role of hyperthermia as a sensitizer and, finally, to correlate the findings to previous reports.

Previously, the observations on the effect of ionizing radiation have always been made after the conclusion of irradiation (in some experiments as long as 30 days afterwards) but in the present experiments the immediate effects were recorded. The influence of temperature on the physiologic activity of epithelial cells (beating ciliated cells) exposed to irradiation was continuously recorded during the exposure.

Methods and Material

The method of HÅKANSSON & TOREMALM (1965) for recording the activity of the tracheal cilia in rabbits, which was shown to allow simultaneous irradiation and recording (BALDETORP *et coll.* 1976), was used.

The trachea from a total of 80 healthy rabbits was used, 20 specimens at each temperature-level. The animals were killed by a blow on the skull. The trachea was immediately dissected and placed in an experimental chamber where even temperature and humidity could be maintained at the desired level. The trachea was then opened by a longitudinal incision in the membranous part and the mucociliary activity was recorded during 30 minutes before irradiation, allowing the trachea to adapt to the conditions in the chamber. This occurred rather quickly, and the constancy in the wave-frequency was maintained satisfactorily. The humidity was kept above 90 per cent in all of the experiments. Irradiation was administered by Philips Contact Therapy apparatus at 50 kV, 2mA, HVL 0.5 mm Al, focus-object distance 40 mm. The dose rate was 0.34 Gy/s. Each tracheal specimen was given 10 Gy. The dose given to the tracheal epithelium was measured using thermoluminescent dosimeters (TLD). The mucociliary activity was recorded continuously during the exposure at four different temperature levels: 20°C, 30°C, 37°C and 40°C.

Results

Irradiation increased the mucociliary wave frequency in all experiments. This occurred immediately during the first 2 to 3 seconds, increased to a maximum lasting for a certain time and then decreased rather slowly. The results are given in Table 1

Table 1

Increase of the mucociliary wave frequency at different temperature levels during the initial 25 seconds of irradiation

Time after start of irradiation (s)	Accumulated dose (Gy)	Increase in per cent (Mean $\pm$ SD)			
		20°C	30°C	37°C	40°C
5	1.7	14.0 $\pm$ 2.5	10.1 $\pm$ 6.3	7.0 $\pm$ 1.5	9.0 $\pm$ 1.8
10	3.4	17.5 $\pm$ 2.0	16.1 $\pm$ 1.2	9.0 $\pm$ 2.0	9.8 $\pm$ 2.0
15	5.1	16.0 $\pm$ 2.5	14.6 $\pm$ 2.5	9.5 $\pm$ 1.5	9.0 $\pm$ 2.5
20	6.8	11.2 $\pm$ 2.5	10.8 $\pm$ 2.4	7.5 $\pm$ 2.0	7.0 $\pm$ 1.5
25	8.5	7.2 $\pm$ 3.0	7.7 $\pm$ 1.5	4.5 $\pm$ 2.0	4.2 $\pm$ 2.0

as mean values in per cent of a reference value at 5, 10, 15, 20 and 25 seconds after the start of irradiation. This reference value was determined for each animal as a mean value of the frequency during 30 seconds immediately before irradiation. The highest values were found at 20°C, except for 25 seconds where the highest value was at 30°C. The results for each temperature level will therefore be commented upon:

20°C: After 5 s, the frequency had gradually increased 14 per cent ($SD \pm 2.5$), at 10 s, the increase was 17.5 per cent ($SD \pm 2.0$) and after 15 s it was reduced to 16 per cent ($SD \pm 2.5$). This decrease continued and at 20 s the increase was 11.2 per cent ($SD \pm 2.5$). Finally, the increase reached 7.2 per cent ($SD \pm 3.0$) after 25 s. The maximum increase was found at 10 s after the start of irradiation, when an accumulated dose of 3.4 Gy had been given.

30°C: The frequency increased 5 s after the beginning of irradiation to a value of 10.1 per cent ($SD \pm 6.3$), at 10 s, it was 16.1 per cent ($SD \pm 1.2$). The increase then reduced to 14.6 per cent ($SD \pm 2.5$) at 15 s, to 10.8 per cent ($SD \pm 2.4$) at 20 s and to 7.7 per cent ($SD \pm 1.5$) at 25 s. The maximum increase of the mucociliary wave frequency was around 10 s (accumulated dose 3.4 Gy). The maximum value was reached about 7 s after the start of irradiation, but remained near this value for another 9 s (Fig. 1).

37°C: The response to irradiation occurred as quickly as at 20°C and at 30°C, but the maximum increase was lower than in the preceding experiments. At 5 s, the increase was 7.0 per cent ($SD \pm 1.5$), at 10 s after the beginning of irradiation, it was 9.0 per cent ($SD \pm 2.0$), at 15 s, 9.5 per cent ($SD \pm 1.5$), after 20 s 7.5 per cent ($SD \pm 2.0$), and at 25 s it was 4.5 per cent ($SD \pm 2.0$). The total response to irradiation was lower at this temperature compared to 20°C and 30°C, but the maximum increase could be estimated to approximately 15 s (accumulated dose 5.1 Gy).

40°C: At this temperature, which is slightly higher than the normal body temperature of the rabbit (37°C), the frequency curve was similar to the curve at 37°C, and a

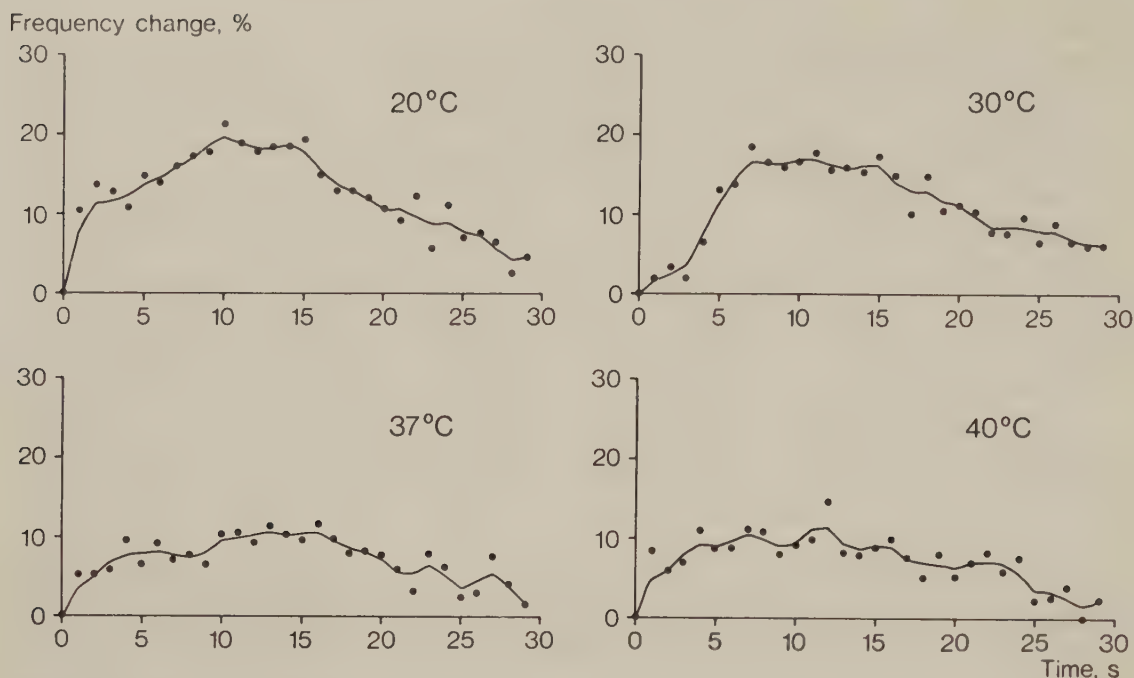


Fig. 1. Changes in mucociliary wave frequency during irradiation expressed in per cent of the reference value.

long lasting moderate plateau occurred. The increase of the mucociliary wave frequency at 5 s after the beginning of irradiation was 9.0 per cent ($SD \pm 1.8$), at 10 s 9.8 per cent ($SD \pm 2.0$), and after 15 s it was reduced to 9.0 per cent ($SD \pm 2.5$). This decrease continued and the increase was 7.0 per cent ($SD \pm 1.5$) after 20 s, and 4.2 per cent ($SD \pm 2.0$) after 25 s. In this experiment the maximum activity was obtained at 10 s (accumulated dose 3.4 Gy).

A second-by-second plotting of the mean values from the four different temperature levels appears in Fig. 1 together with the curves as they were calculated by a computer. It is evident that: (1) The greatest percental increase of the mucociliary wave frequency is obtained at low temperatures (20°C and 30°C), (2) all of the curves have their maximum at about the same time after the start of irradiation ($\sim$ the same absorbed dose), and (3) if the curves are divided into two groups: a low-temperature group (20°, 30°C) and a high-temperature group (37°, 40°C), the values in the first group are always higher than those in the second group.

A closer analysis of the curves was made in an attempt to explain the findings. The k -value (slope) for a regression line based on the percental increase of the mucociliary wave frequency as calculated second-by-second has been estimated for the 0 to 10 seconds interval and for the 10 to 20 seconds interval after the start of irradiation (Fig. 2). At 20°C, the k -value was calculated to 1.4 (time-interval: 0 to 10 s) and to -1.0 (time-interval: 10 to 20 s). The corresponding k -values at 30°C were 2.0 and -0.6 , respectively, and at 37°C 0.6 and -0.3 . At 40°C, the value was 0.6 in the initial phase and -0.5 in the second phase.

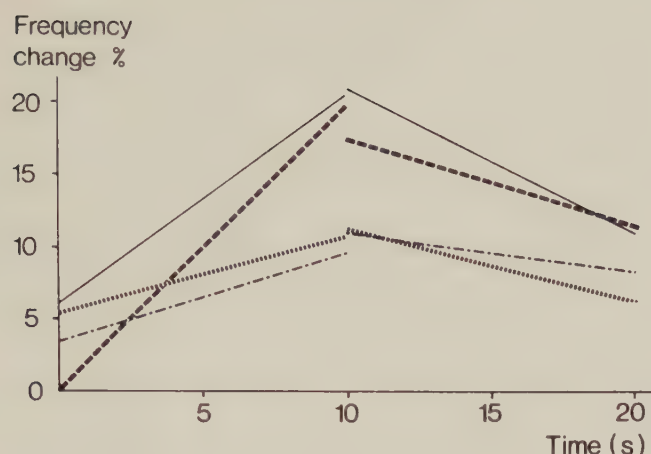


Fig. 2. K-values of the four different curves in Fig. 1. Low temperatures have the highest values. — 20°C, --- 30°C, - · - · - 37°C, 40°C.

From the curves in Fig. 1 it is possible to determine the exact maximum points. The values are given in Table 2. The maximum response was found at an accumulated dose of 3.4 to 4.4 Gy, the highest value coinciding with the normal body temperature of the rabbit.

When the cilia are beating they perform normal steady-state work. When this balance is changed, e.g. during irradiation, and the beating is intensified (Fig. 1), the increased work during irradiation must be proportional to the area limited by $x=0$ and $x=28$ (~ 10 Gy). It is then assumed that this area is an expression for the amount of energy made available to the cilia by the irradiation. The nearest approximation for calculating the areas described by the curves is to use SIMPSON's formula:

$$\int_a^b f(x) dx = \frac{b-a}{3n} (f_0 + 4f_1 + 2f_2 + 4f_3 + \dots + 2f_{n-2} + 4f_{n-1} + f_n) - \frac{(b-a) \cdot h^4}{180} f^{IV}(\xi),$$

$a < \xi < b$

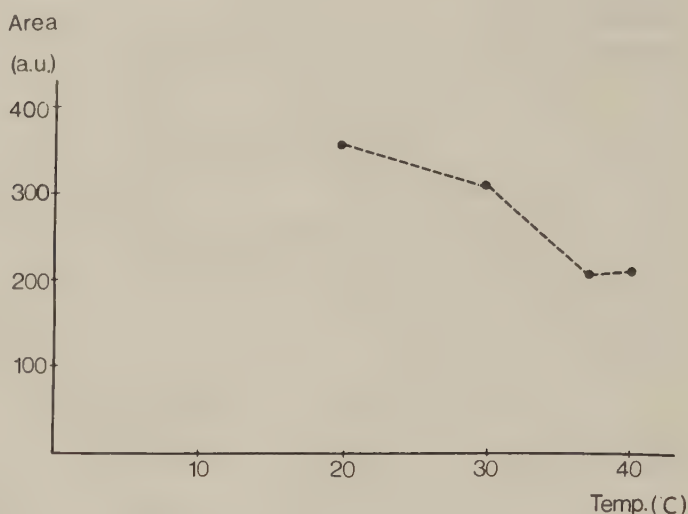
where $a=0$ and $b=28$ constitute the limits of integration. n is the number of partial intervals, h , having the length of 1. The formula postulates n to be an even figure.

Table 2

The accumulated dose at the different temperatures, calculated at the maximum point from the polynomial function of the curves in Fig. 1

Temperature (°C)	Accumulated dose at max. response (Gy)
20	3.4
30	3.6
37	4.4
40	3.7

Fig. 3. Integration of the function of the four curves in Fig. 1, to $x=28$ seconds, plotted against temperature. The size of the surfaces constitutes an indirect measure for the amount of released ATP which has been available as a source of energy for the cilia (i.e. which has not been transphosphorylated to FDP). Area in arbitrary units (a.u.).



f_0 is the functional value of the time 0 s

f_1 is the functional value of the time 1 s

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f_n is the functional value of the time n s

The last term: $((b-a) \cdot h^4/180)f^{IV}(\xi)$ is an error factor which may be eliminated in the present case, since the purpose of integration is for comparing the curves only.

If the calculated area is plotted against the temperature (Fig. 3), it is seen that the lowest temperatures (20°C and 30°C) have the largest areas. The slope for a regression line based on the four area-values is -8.2 .

Discussion

The mucociliary activity in all experiments increased during irradiation at the four temperature levels: 20°C, 30°C, 37°C, 40°C. This increase occurred within 5 seconds after the start of exposure. This phenomenon at 30°C has been described previously (BALDETORP et coll.). Now, a refined second-by-second analysis of the previous findings is presented together with the evaluation of the present results.

The following facts can be established (Fig. 1):

(1) The radiation effect in the initial phase (first 10 s) varied with temperature. At low temperatures (20°C and 30°C), the increase in the mucociliary activity versus time is greater than at high temperatures (37°C, 40°C). The k -values (Fig. 2) clearly show the difference.

(2) In the second phase (10 to 20 s) principally the same behaviour exists during the declination of the frequency increase, but the k -values at the low temperatures have a sharper slope than at the higher ones (Fig. 2).

(3) The peaks or plateaus of the curves (Fig. 1) are higher at 20°C and 30°C than at 37°C and 40°C.

The conclusion to be drawn must be that the initial response of the mucociliary activity to ionizing irradiation is greater at low (20°C to 30°C) temperatures than at normal or high (37°C to 40°C) temperatures. This surprising finding would seem to contradict the current assumption that irradiation at high temperature is more effective than at low temperature. ROBINSON *et coll.* (1974) irradiated normal mouse skin and C3H mammary tumours at temperatures from 37.5°C to 43.0°C. They reported an increasing thermal enhancement ratio (TER) in relation to increasing temperature. On the contrary, the present results seem to indicate that hyperthermia does not influence the early effects of irradiation.

No experiments seem to have been made to analyse the effect of ionizing irradiation on the cell during treatment. Previous authors have recorded the late effects: the survival and death of the cells. Therefore, their conclusions cannot be directly applied to the present experiments and there need not to be any conflict between the opinions.

In order to explain the present findings, it is necessary to establish a hypothesis for the energy principle of the physiologic activity produced by the ciliated cells. It must be reasonable to assume that adenosine triphosphate (ATP) is the energy source for the ciliary movements (SATIR 1974). ATP is produced in the mitochondria and, as demonstrated morphologically by the electron microscope, the mitochondria are concentrated in the apical part of the ciliated cells (BALDETORP *et coll.*, to be published). This localization near the surface makes the mitochondria a vulnerable target for irradiation. Furthermore the close relation to the ciliary basal bodies means that the diffusion distance for the ATP is very short. Previously (BALDETORP *et coll.*) it was assumed that ATP was made accessible to the beating cilia through the influence of the ionizing radiation on the permeability of the mitochondrial membranes. This hypothesis is supported by the present finding and the experiments show furthermore that more ATP must be available as a source of energy at 20°C to 30°C than at 37°C to 40°C. It is assumed that the actual release of ATP is not necessarily influenced by the temperature.

The effect of radiation on the glycolysis of rat thymocytes was investigated by OHYAMA *et coll.* (1967). They exposed a suspension of thymocytes to 8000 R, and determined the intermediate products of the glycolytic process. At one and two hours of incubation following irradiation they found a significant change in the steady-state of the intermediate products of the glycolysis: the concentration of fructose-1,6-diphosphate (FDP) was highly increased. From these results it was deduced that the irradiation had a specific facilitation influence on the activity of the enzyme phosphofructokinase (FPK).

OHYAMA *et coll.* (1974) were able to prove that the content of ATP in thymocytes was reduced after irradiation. This reduction was found to be related to the absorbed dose and to the incubation temperature. As the ATP reduced due to the absorbed

dose and to temperature, a corresponding increase of the concentrations of fructose-1,6-diphosphate (FDP) and dihydroxyacetone-phosphate (DHAP) occurred. They found the processed intermediates to be marked at 37°C, but at 25°C the changes in the steady-state concentration of the intermediates were small, indicating inactivation of FPK at 25°C. As a whole the reaction may be summarized in the formula:



where phosphofructokinase catalyzes the reaction depending on the temperature. The irradiation is assumed to enhance the activity of the enzyme FPK. Thus, when ATP is transphosphorylated to FDP the energy pool available to the cilia must diminish in relation to the increase in temperature to a certain level. The catalytic activity of FPK is consequently assumed to be more and more inhibited as the temperature is reduced. This is confirmed in Fig. 3 and the conclusion to be drawn is that more work is performed at lower temperatures, and that more energy (ATP) must be available at the low than at the high temperatures. It is also reasonable to believe that the enzymatic activity ceases at a certain level. The rate at which the transphosphorylation of ATP to FDP took place was reported by YAMADA & OHYAMA (1970) to be high, e.g. in only minutes after irradiation. However, they could not detect the reaction during the irradiation. The present experiments seem to indicate a reaction which takes place within seconds after the start of irradiation. The present results correspond well to the theories of OHYAMA et coll., and not only a release of ATP must be accepted, but also a possible stimulation of the phosphofructokinase during the initial phase of irradiation.

Before this hypothesis can be presented as a theory the importance of the viscosity of the mucus has to be taken into consideration. In the non-irradiated trachea, the mucociliary activity is obviously lower, the lower the temperature (MERCKE et coll. 1974), and a possible explanation for this is the change in viscosity of the mucus. Under these conditions, the percental increase of the mucociliary activity at irradiation would follow a curve which could be based on the values given by MERCKE et coll. However, the present experiments would seem to contradict such a statement. The increase of the activity was about the same at 20°C as at 30°C. The same appeared for the temperature range 37°C to 40°C. The difference obviously supports the fact that changes in viscosity in the mucus in relation to temperature play a less important role in the evaluation of the results of the present experiments.

In conclusion, the results have shown that the early effects of radiation, recorded as changes in the physiologic activity of ciliated cells, are thermally dependent. The hypothesis regarding ATP-release and the action of phosphofructokinase during irradiation is supported by OHYAMA et coll. In contrast to the late radiation effects, which are reported to be potentiated by hyperthermia, no such phenomenon could be demonstrated in the initial effects of irradiation. In spite of this discrepancy, there is no reason to doubt the hyperthermal dependency of the late radiation effects.

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SUMMARY

The influence of temperature on the early effects of ionizing irradiation has been analysed using the trachea of the rabbit. ATP is assumed to be released by the effect of irradiation on the mitochondrial membranes, which increases the mucociliary activity in the epithelium, registered second-by-second during irradiation performed at 20°C, 30°C, 37°C and 40°C. The early effects of irradiation depended on the temperature, while hyperthermia does not potentiate this condition. A possible explanation is that phosphofructokinase, which is probably stimulated by irradiation, is inactivated at lower temperatures.

ZUSAMMENFASSUNG

Der Einfluss der Temperatur auf die frühzeitigen Wirkungen von ionisierender Strahlung unter Verwendung der Trachea des Kaninchens wurde analysiert. Es wird vermutet, dass ATP freigesetzt wird durch Effekte der Bestrahlung auf die Membranen der Mitochondrien, was zu einem Anstieg der Mucociliaraktivität des Epitels führt, welche Sekunde für Sekunde während der Bestrahlung bei 20°C, 30°C, 37°C und 40°C registriert wurde. Die frühzeitigen Wirkungen der Bestrahlung hängen von der Temperatur ab, während Hyperthermie diese Veränderungen nicht potenziert. Eine mögliche Erklärung ist, dass Phosphofructokinase, die wahrscheinlich durch Bestrahlung stimuliert wird, bei niedrigen Temperaturen inaktiviert ist.

RÉSUMÉ

Les auteurs ont examiné l'influence de la température sur les effets précoces des radiations ionisantes sur la trachée du lapin. Ils pensent que de l'ATP est libéré sous l'effet de l'irradiation sur les membranes des mitochondries, ce qui augmente l'activité mucociliaire de l'épithélium enregistrée seconde après seconde pendant l'irradiation effectuée à 20°C, 30°C, 37°C et 40°C. Les effets précoces de l'irradiation dépendent de la température alors que l'hyperthermie ne potentialise pas ce phénomène. Une explication possible est que la phosphofructokinase, qui est probablement stimulée par les radiations, est inactivée à des températures plus basses.

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INTRACELLULAR RECORDING OF POTENTIAL OSCILLATIONS IN CILIATED CELLS EXPOSED TO IONIZING RADIATION

B. BALDETORP, L. BALDETORP and C. H. HÅKANSSON

Abstract

Intracellular potential oscillations of the ciliated cells of the rabbit's trachea have been measured by means of glass capillary microelectrodes during exposure of the cells to ionizing radiation. The electrophysiologic activity was fairly stable during the entire exposure at a total administered dose of 10.0 Gy. This finding is contradictory to the change in mucociliary activity obtained under the same conditions when measured with a light beam reflex method, but is in agreement with the findings regarding the influence of radiation on the membrane potentials as far as nerves and muscles are concerned.

Experiments concerning the motility of cilia exposed to ionizing radiation have been performed since the beginning of this century. Some reports indicate that ionizing radiation acts as a stimulus on ciliary activity (12, 27, 28). Other investigations indicate, however, that the motility of ciliated cells is reduced after exposure to radiation (9, 24).

The physiologic activity of cells is able to change immediately on radiation. BALDETORP and his co-workers (1-5) showed *in vitro* that ciliated cells of the rabbit's trachea increased their beating frequency during the beginning of exposure to radiation, and this was assumed to be an effect of stored adenosine triphosphate (ATP) released as a result of radiation. The increased amount of ATP due to damage of the membranes of the mitochondria would then instantaneously act on the ciliary motility.

On the basis of the 'pace-maker' theory proposed by HÅKANSSON & TOREMALM (11), it was found logical to investigate the effect of ionizing radiation on the electrophysiologic activity in the ciliated cells of the rabbit's trachea during irradiation and to compare the results with the ciliary beat frequency as measured in the investigation reported by BALDETORP *et coll.* (5).

Material and Methods

The tracheas from 20 healthy rabbits weighing 1.5 kg were used. The animals were killed by a blow on the skull. Thereafter a 30 mm piece of the trachea was immediately dissected and fibrous tissue was removed from the specimen. The tracheal specimen was stretched 15 to 20 per cent from the collapsed state to its original length and mounted in an experimental chamber (Fig. 1). The temperature and air humidity were kept at 37°C and above 90 per cent, respectively. The trachea was then opened by an incision, 20 mm in length, in the membranous part and left to adapt to the chamber conditions during 30 minutes. After the phase of adaptation the intracellular activity of one ciliated cell was recorded by a capillary glass microelectrode, filled with 3 M KCl solution (13). The signals from the electrode were fed into a transistorized impedance converter with

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an input impedance above 10^9 ohm and thereafter into an oscilloscope (Tektronix 565) with a differential amplifier (Tektronix 3A 3). The impedance and the tip-potential of every electrode were measured and allowed to be 10^7 ohm and less than 5 mV, respectively.

In order to remove the noise generated by the electronic equipment the amplified signal was filtered in an active electronic filter (Krohn-Hite 3550) put in band-pass mode and with the filter transmission band adjusted to 3 to 30 Hz. Finally, the filtered signal was recorded on an ink-writer (Mingograf 34, Elema-Schönander). The method and electrical apparatus have been described in detail by HÅKANSSON & TOREMALM (11), and with modifications by TOREMALM et coll. (26).

Procedure. The microelectrode, mounted on a micromanipulator holder, was carefully moved down to the mucosal layer until the tip of the electrode was in contact with the secretion layer. Before the established contact, the signal, registered on the oscilloscope screen, was irregular and floating, while in the contact state the signal became stabilized to the reference level according to the junctions in the whole system. The microelectrode was then forced through the mucous until the membrane of a ciliated cell was penetrated. This could be seen on the oscilloscope which at first was set to DC-mode. If a potential of -25 mV to -40 mV was obtained the electrode could be regarded as being in the ciliated cell. Cells with a negative membrane potential of not less than 25 mV were accepted for the experiments. Lower values (more positive) were excluded because of the risk of their being in an injured cell.

The oscilloscope was then shifted to AC-mode and the gain of the differential amplifier was increased in order to visualize the potential oscillations on the screen. Sometimes the potentials (oscillations) disappeared from the oscilloscope screen but returned after some seconds. This was interpreted as the result of the rhythmically contracting smooth muscles of the trachea causing the electrode tip to be retracted from the cell or to penetrate it. To eliminate this phenomenon the trachea had to be expanded more and the experiment had to be started again. With the electrode in the ciliated cell the electrical activity was recorded during 30 seconds and then the specimen was irradiated by a Philips' contact therapy unit at 50 kV, 2 mA, HVL 0.5 mm Al, focus-object distance 60 mm, dose rate 0.15

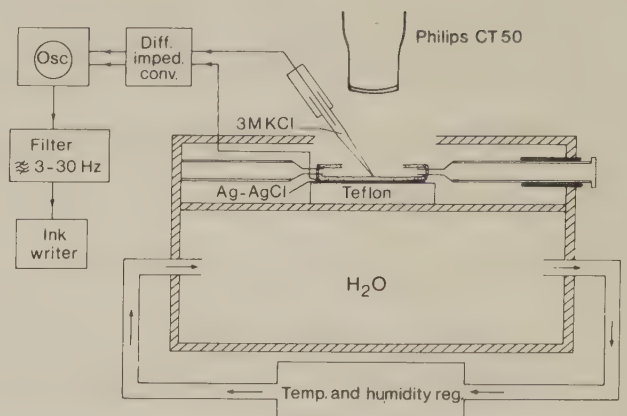


Fig. 1. Experimental chamber for simultaneous irradiation and recording of the intracellular electrical activity.

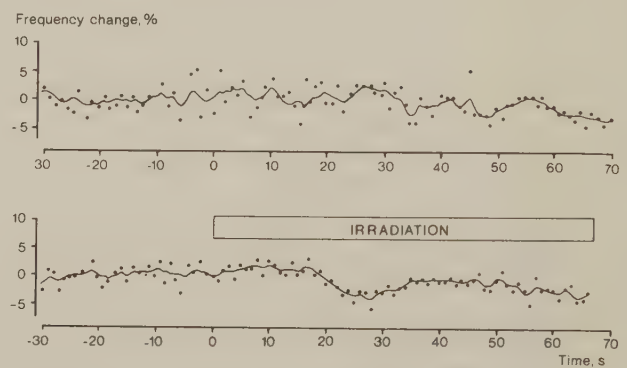


Fig. 2. a (upper): Frequency change in the intracellular potential oscillations of non-irradiated ciliated cells. b (lower): Percentual changes in the frequency of the intracellular potential oscillations of ciliated cells during irradiation.

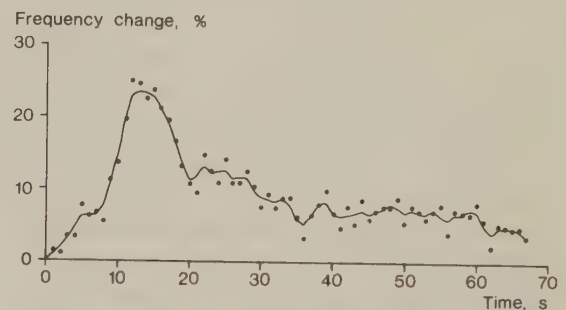


Fig. 3. Percentual changes in the mucociliary wave frequency during irradiation (Baldetorp & Håkansson 1977).

Gy/s. The recording continued simultaneously with the irradiation until the preparation had absorbed a dose of 10 Gy. The irradiation given to the tracheal epithelium was measured by using thermoluminescent dosimeters (TLD) placed on both sides of the tracheal wall.

Table

Mean values of the intracellular activity for each five second period after the start of irradiation during the entire exposure

Time after start of irradiation (s)	Accumulated dose (Gy)	Intracellular activity (osc/s $\pm$ SD)
Mean value before irr.	—	18.7 $\pm$ 0.21
5	0.75	19.0 $\pm$ 0.26
10	1.50	19.0 $\pm$ 0.20
15	2.25	18.9 $\pm$ 0.19
20	3.00	18.8 $\pm$ 0.24
25	3.75	18.3 $\pm$ 0.32
30	4.50	18.1 $\pm$ 0.21
35	5.25	18.4 $\pm$ 0.17
40	6.00	18.5 $\pm$ 0.05
45	6.75	18.6 $\pm$ 0.10
50	7.50	18.5 $\pm$ 0.25
55	8.25	18.1 $\pm$ 0.44
60	9.00	17.8 $\pm$ 0.22
65	9.75	17.9 $\pm$ 0.21

Results

The intracellular potential oscillations varied in the tracheal specimens from 15 to 20 Hz. The mean value of the frequency of the potential oscillations during the 30 seconds immediately before irradiation constituted the reference value for each experiment. In all experiments the frequency recorded during the irradiation was every second percentually related to the corresponding reference level. A computer made up a plotting based on these values from the 15 experiments as a function of the time of exposure.

Fig. 2b shows the pre-irradiation period of 30 seconds and the frequency changes during 67 seconds of irradiation to 10 Gy. Irradiation did not seem to have any influence on the frequency of the potential oscillations as compared with those from non-irradiated cells (Fig. 2a). Here the result originated from five control experiments during 97 seconds from non-irradiated cells. In the individual cells, the frequency was mostly constant during this time. In some experiments a tendency to a slower activity at the end of the measurement was noted.

The mean values with standard deviations of the percentage of the frequency for each five second period during the entire exposure are shown in the Table. As in the non-irradiated controls, the frequency in the beginning was constant. During irradiation the frequency was slightly more irregular but

the changes were within 5 per cent of the reference value. A small decrease from 19.0 to 17.8 Hz (Table) was noted but this was not caused by the irradiation since it was also seen in the controls. It was interpreted as a result of potassium leakage at the tip of the electrode. The same explanation was made regarding the amplitude of the oscillations.

For comparison the dependence of the mucociliary activity on ionizing radiation as measured extracellularly is shown in Fig. 3. This diagram originates from earlier experiments (2) with the same conditions as in the present investigation. Each point in the plotting corresponded to the mean value of 20 animals. A rapid increase was observed and the mucociliary activity reached a maximum increase at about 12 to 15 seconds, i.e. at an absorbed dose of about 1.5 Gy. Thereafter a slight decrease in the activity to the original level followed.

Discussion

Basic studies by HÅKANSSON & TOREMALM (11) concerning the electrophysiology of the ciliated cell of the rabbit's trachea showed that the cell has a membrane potential of approximately -40 mV and a periodically fluctuating AC-component of 1 mV. This oscillating component was considered to function as a 'pace-maker' in order to control the ciliary beats (11). YANAURA et coll. (29) measured a membrane potential of -28.6 mV with an oscillating component of 0.2 mV in the canine tracheal ciliated cell, and they concluded that the electrical activity is closely related to the mechanical activity in the beating cell.

Earlier investigations have indicated that ionizing radiation is able to change the ciliary beat frequency. BALDETORP et coll. (3–5), 1976, 1977 a, b), BALDETORP & HÅKANSSON (2) and BALDETORP (1) observed that ciliated cells are able to react immediately with 20 to 30 per cent increased beating frequency when exposed to 1.0 to 1.5 Gy. No one, as far as is known, has measured the membrane potential of a ciliated cell during irradiation, and therefore the present experiments were carried out in order to elucidate whether the intracellular conditions in the cell, i.e. the membrane potential and 'pace-maker' frequency, react in the same manner corresponding to the results reported by these authors.

The present results indicated that the frequency of the potential oscillations was fairly stable during the whole exposure up to 10 Gy. Changes in tem-

perature did not affect the internal electrical oscillations either, as reported by MERCKE et coll. (21). The beating of cilia, however, is greatly affected but the divergence between the two parameters as the temperature is lowered was explained by the increase in tenacity of the mucous medium into which the cilia are beating. The resistance to low doses of ionizing radiation is also apparent in nerve cells and muscle cells. NACHMANSOHN (22) noticed reduced speed of the action potential in the irradiated squid giant axon. This phenomenon, however, was obtained only after very high doses (50 kR). BERGEDER (6, 7) obtained similar results when irradiating frog muscles with 10 to 50 kR. In addition, he recorded a membrane potential lower than normal, caused by a leakage of potassium through the membrane. Lower daily doses as used in clinical cases (2.0 Gy) do not affect electrophysiologic activity, e.g. the EEG at irradiation of the brain, and ECG during irradiation of the heart in patients with Hodgkin's disease or lung cancer (personal observations). These findings are therefore in accordance with the present experiments, where no change of the electrical activity was obtained during irradiation up to a dose of 10.0 Gy.

As a comparison, drugs have been shown to influence the electrical activities of the canine tracheal ciliated cells (29). The amplitude and the frequency of the potential oscillations were increased by acetylcholine and adrenaline while dibucaine, lidocaine and 5-HT caused a decrease of the two parameters measured.

Investigations on lower ciliated organisms indicate an intimate connection between the electrical activity of the cell and the ciliary motility (8, 15–20, 23). HORRIDGE (14) found action potentials in *Ctenophora* ciliated cells, fired with the same frequency as the ciliary beating frequency. The regulatory mechanism, however, seems to be complicated. In *Paramecium* and *Stylonychia* the described events could also be related to the difference in the concentration of Ca^{++} across the cell membrane in such a way that an influx of Ca^{++} -ions may cause the normal stroke to be reversed and the frequency of beating to increase. This can be provoked by mechanical stimuli which also have an effect on the K^{+} -dependent potential.

Mechanical stimulation at the anterior end seems to provoke the Ca^{++} -depolarisation factor and mechanical stimulation at the posterior end to affect the K^{+} -hyperpolarisation factor (25), also with an

increase of the beat frequency in the normal direction.

The initial effect of ionizing radiation seems difficult to explain. The ciliary beating reacts immediately with an increase in frequency as soon as the radiation is applied and reaches a peak of about 20 to 30 per cent above the reference value at an absorbed dose of 1.0 to 1.5 Gy, independently of the dose rate (1–5). The high increase, however, had a duration of only 10 to 15 seconds and was explained as the effect of an ATP release caused by influence of the irradiation on the cell membrane or of the membranes of the organelles (mitochondria; (1–5, 12, 24). The increase of the beat frequency in the present experiments was not accompanied by an increase of the intracellular oscillations during radiation. As a matter of fact, the electrical frequency could be regarded as nearly constant. As far as is known, the influence of ionizing radiation on the electrical activity of the ciliated cells during exposure has not been described.

In conclusion, the real connection between the electrical activity as measured inside the cell and the beating of cilia as measured extracellularly is complicated and further experiments must be undertaken, especially regarding the effect of irradiation on the sodium pump, potassium pump and calcium pump.

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**A COMPARISON BETWEEN
THE IMMEDIATE EFFECTS OF
PHOTON AND ELECTRON RADIATION
ON THE CILIARY ACTIVITY
OF THE RABBIT'S TRACHEA**

An In Vitro Study

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SUMMARY

The immediate effects of 6 MV X-rays and 4 MeV electron-rays on the mucociliary activity of the trachea of the rabbit were studied. In spite of the same average LET for the two radiation qualities, there was a clear difference in their effects on the ciliary motility during irradiation. The increase of the mucociliary activity after an absorbed dose of 1.5 Gy X-ray reported earlier was confirmed by the present investigation when using 6 MV photons. Electron radiation increased the beat frequency to a corresponding degree already at an administered dose of 0.5 Gy. The differences found in the pattern of the mucociliary activity during irradiation are discussed. The results indicate that the conventional RBE-values cannot be used for early physiological effects of irradiation.

Key-words

Tracheal epithelium; Ciliated cells; Cilia; Radiation; Photons; Electrons.

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INTRODUCTION

The ciliated epithelium of the trachea has proved to be an almost unique test object for investigating the early (e.g. during irradiation) and the late (i.e. hours or days after irradiation) effects of ionizing radiation. In a study of the physiological activity of the cilia (their beat frequency), Baldetorp et coll. (1976) were able to demonstrate an increased beat frequency when their preparations were exposed to radiation. This increase was usually between 20 and 25 per cent. These findings again raised the well-known question in radiobiology as to the stimulation induced by radiation.

It was assumed that this increase of the beating frequency was the result of influence on the ATP-producing mitochondria of the ciliated cells. Although the underlying mechanism of this phenomenon is not fully understood, the results of low-voltage X-rays (50 kV) and those of gamma radiation from a cobalt-source have always been a stimulation of the beating frequency, and this has been valid for different dose rates, as well. As to the immediate reaction following irradiation Fujiwara et coll. (1972) found a decrease of the activity in the respiratory tract by single doses up to 30 Gy, which was restituted within two hours.

No experiments have thus far been conducted with photon energies higher than ^{60}Co or with high energy electrons, but since their use in clinical radiation therapy is of increasing importance, experiments are called for to gain information about their interaction with matter.

The present study has aimed at investigating the effects of 6 MV photons on the ciliary beat frequency, and to compare these effects with those of 4 MeV electrons. As in earlier investigations, the focus of interest has been limited to the events during irradiation, as these studies touch on a controversial problem within radiobiology - radiative stimulation.

MATERIAL AND METHODS

The trachea from 20 healthy rabbits was used, ten animals being assigned to each radiation quality. They were sacrificed by a blow on the head, after which the trachea was immediately dissected and placed into the experimental

chamber, which had a temperature of 37°C and a humidity of more than 90 per cent. The specimens, which were about 30 mm in length, were placed in the manner described by Håkansson & Toremalm (1965), and stretched to their original length (15-20 per cent more than in the collapsed state). They were then opened by an incision in the membranous part (length 20 mm), and left to adapt to the chamber conditions during 30 minutes. The recording of the beating cilia was made according to the method of Baldetorp et coll. (1976), which permits examination during the irradiation of the ciliated epithelium. Before starting irradiation, it was first ascertained that the beat frequency was stable, i.e. that the specimen had been adapted to the conditions in the chamber. The beat frequency was then registered continuously during 30 s prior to the treatment.

Irradiation was performed with two different qualities:

I. Photons generated at 6 MV from a linear accelerator (Siemens Mevatron 6) with FFA = 600 mm, and the field size 100 x 100 mm at the focus-object distance 680 mm. In order to get an improved roentgen-ray spectrum with regard to high-energy photons, a Cu-flattening filter was utilized in the linear accelerator. The filter had a high absorption coefficient for low-energy photons up to 2 MV. Absorption was reduced at higher energies, with a minimum at 8-9 MV.

The dose rate was 0.07 Gy/s at the tracheal mucous membrane. The dosimetry was performed with thermoluminescent dosimeters (TLD) at the same place as the tracheal specimen in the experimental chamber. The deviation from the calculated dose rate was within 3.5 per cent. Each tracheal specimen received 10 Gy.

II. Electrons with an energy of 4 MeV from a linear accelerator (M.E.L. SL 75, Philips). With an electron therapy applicator mounted on the treatment head, the field size was 100 x 100 mm at a focus-object distance of 1080 mm. A thin aluminium foil was inserted in the treatment head in order to absorb scattered electrons from the main beam. The dose rate was 0.05 Gy/s at the tracheal mucous membrane. Dose measurements were carried out with plane parallel ionization chambers. Each tracheal specimen received 5 Gy.

RESULTS

I. Photon radiation

During the 30 seconds prior to the start of irradiation, the beat frequency varied in the different specimens from 14.5 to 22.5 beats/s, when calculated second-by-second. Despite this difference in beat frequency, the frequency increase during the irradiation was nearly the same for all specimens. In the single specimen, the standard deviation (SD) was 1.0 beats/s, but for the whole material, the SD was 2.5 in per cent. The mean value of these pre-radiation measurements constituted the reference value (zero-level) for the further measurements. Each specimen was, thus, its own control.

The mean values of the frequency change for each five-second period after the start of irradiation are given in Tables 1 and 2.

The percentual changes of the beat frequency for 6 MV photons are shown in the Figure. During the first 15 seconds after the onset of radiation the level of the beat frequency increased with 4-5 per cent, which was statistically significant ($p < 0.0002$). This period was called the initial phase. Later, when the trachea had accumulated a dose of about 1.0 Gy, there was a rapid increase (during 5 s) of the beat frequency up to a maximum of about 20 per cent. The accumulated dose was then 1.75 Gy (see the Figure and Table 1). This increased level was maintained for about 5 seconds. During the continued radiation, the frequency change decreased during the following 30 seconds to a value similar to the increase during the "initial phase" of 15 seconds (5%) (see the Figure). This increase remained constant during the whole exposure time of 143 seconds (Table 1), and was statistically significant ($p < 0.0002$).

II. Electron radiation

During irradiation with electrons the beat frequency increased in the same way as during the photon irradiation, but the initial phase lasted only 5 seconds. The maximal increase during this period, however, was the same and also statistically significant. The initial phase was followed by another increase much more rapid than for photons, but the maximal peak was somewhat lower (18%) (see the Figure). This peak was reached after 10 seconds

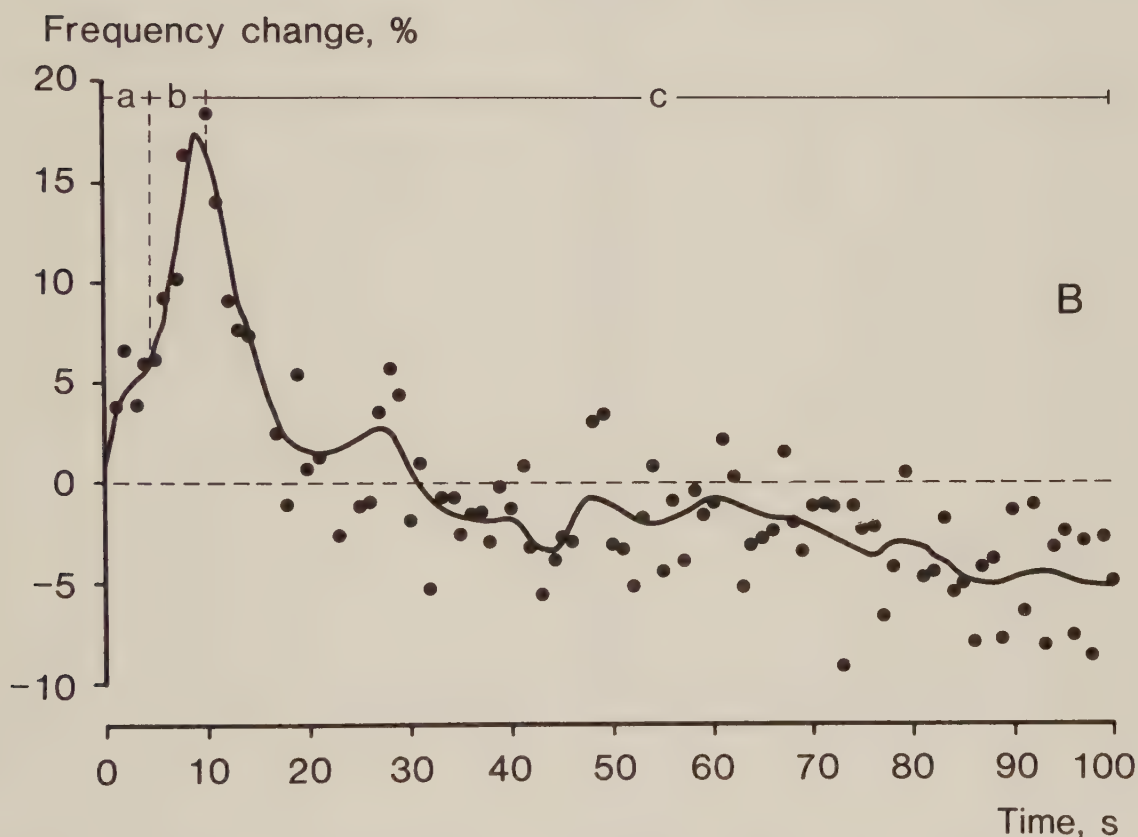
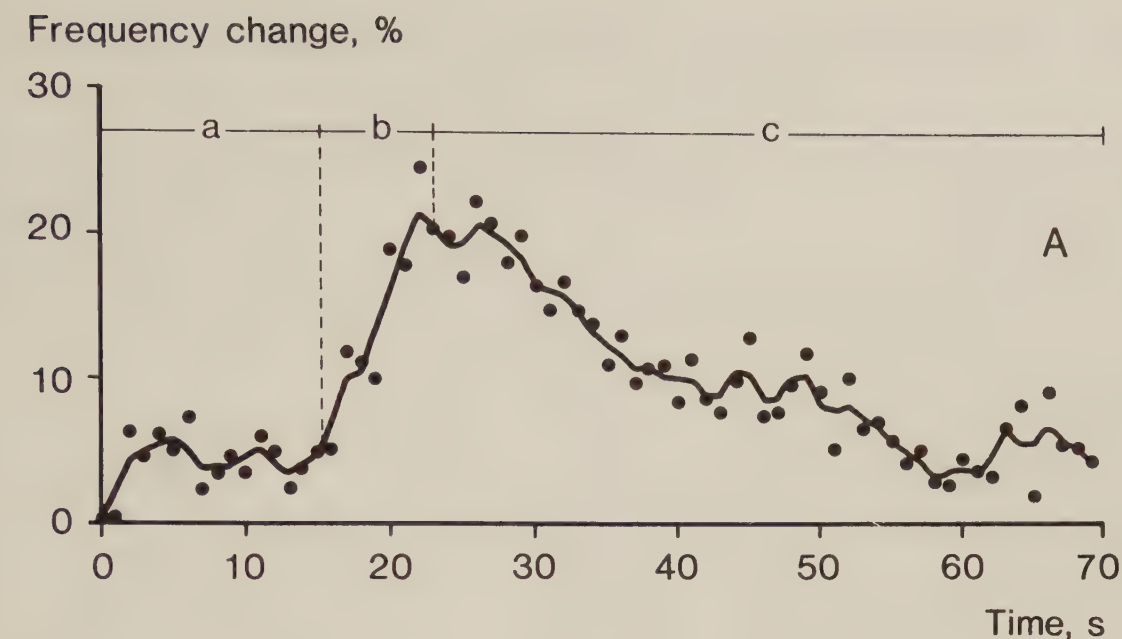


Figure. Changes in beat frequency during irradiation. A) 6 MV photons, 0.07 Gy/s. B) 4 MeV electrons, 0.05 Gy/s. Each point represents the mean value from 10 specimens expressed in per cent of individual reference values established before irradiation. The smoothed curve was calculated by a computer. a = The initial increase. b = The delayed increase. c = The decrease.

Table 1. Mean values for the increase of the mucociliary activity during irradiation with photons 6 MV. (n=10).

Time after start of irradiation (s)	Accumulated dose (Gy)	Increase of muco- ciliary activity (%±SD)
5	0.35	4.5 ± 2.1
10	0.70	4.2 ± 1.7
15	1.05	5.7 ± 3.5
20	1.40	13.7 ± 7.2
25	1.75	20.0 ± 2.3
30	2.10	17.8 ± 2.6
35	2.45	13.7 ± 2.5
40	2.80	10.4 ± 2.1
45	3.15	9.3 ± 1.7
50	3.50	8.9 ± 2.4
55	3.85	6.3 ± 2.3
60	4.20	4.4 ± 1.5
65	4.55	6.2 ± 2.5
70	4.90	6.1 ± 2.2
75	5.25	5.6 ± 1.3
80	5.60	6.9 ± 2.9
85	5.95	7.4 ± 3.0
90	6.30	7.3 ± 2.4
95	6.65	6.6 ± 2.4
100	7.00	6.1 ± 1.4
105	7.35	5.0 ± 2.0
110	7.70	4.5 ± 2.9
115	8.05	5.9 ± 2.0
120	8.40	8.6 ± 1.5
125	8.75	6.3 ± 1.6
130	9.10	5.5 ± 2.3
135	9.45	5.7 ± 2.4
140	9.80	4.7 ± 1.6

Table 2. Mean values for the increase of the mucociliary activity during irradiation with electrons 4 MeV. (n=10).

Time after start of irradiation (s)	Accumulated dose (Gy)	Increase of muco- ciliary activity (%±SD)
5	0.25	8.8 ± 5.0
10	0.50	12.5 ± 4.1
15	0.75	6.5 ± 4.5
20	1.00	2.4 ± 3.2
25	1.25	2.3 ± 3.4
30	1.50	0.6 ± 3.5
35	1.75	-1.6 ± 1.8
40	2.00	-2.1 ± 1.9
45	2.25	-1.1 ± 3.1
50	2.50	-0.9 ± 3.0
55	2.75	-2.3 ± 2.0
60	3.00	-1.5 ± 2.2
65	3.25	-1.5 ± 2.4
70	3.50	-2.1 ± 2.9
75	3.75	-3.0 ± 3.1
80	4.00	-2.6 ± 3.7
85	4.25	-5.0 ± 1.9
90	4.50	-4.9 ± 2.8
95	4.75	-4.7 ± 2.9
100	5.00	-5.3 ± 2.7

of irradiation, corresponding to an absorbed dose of 0.5 Gy, and lasted only for a few seconds. The frequency change then decreased to the zero-level after an absorbed dose of 1.5 Gy (30 s). The activity steadily decreased during the remainder of the exposure, and during the final part of treatment it was 5 per cent below the reference level (see the Figure and Table 2). The total dose absorbed during this time (100 s) was 5.0 Gy.

DISCUSSION

The discussion will first deal with some factors of importance for the interpretation of the results: total absorbed dose, physical differences between the electrons and photons and their interaction with matter, and energy consumption of the ciliae.

The results obtained with both 6 MV photons and 4 MeV electrons agree quite well with those reported by Baldetorp et coll. (1976, 1977) and by Baldetorp & Håkansson (1977), i.e. there was a stimulation of the beat frequency during the entire course of photon irradiation, or during some part of it. The appearance of this stimulation was, however, quite different. During photon irradiation the maximal increase came after an absorbed dose of 1.75 Gy (25 s). The frequency had actually increased significantly (5%) during the first 15 seconds, but the great increase came suddenly, as if caused by some triggering mechanism. During electron irradiation, the course of events was much more rapid and occurred at a lower total absorbed dose. In principle, the reaction during electron irradiation took place at an absorbed dose only one-third of that observed during photon irradiation. The difference in the curves in regard to the maximal increase was, however, not significant. Another important phenomenon was that the decrease during electron irradiation came earlier than during photon irradiation, and that it furthermore dropped significantly below the zero-level.

Photons are indirectly ionizing, i.e. the Compton effect is mainly responsible for producing free radicals and ions, of which the radicals are chemically active and are known to exert a destructive influence.

Electrons are on the other hand directly ionizing and produce the same chemical reagents as photons. In clinical applications, the RBE-factor is

considered to be the same for both radiation qualities. Determinations of RBE-values are, however, based on late effects on cultivated cells or on animal tissues several days after irradiation. Such RBE-factors are therefore inapplicable in the present case, when the radiation interacts with a physiological activity during irradiation.

LET is the energy released per micron of medium (keV/μ). High LET radiation generally produce more damage to the cells than low LET radiation. RBE will usually increase with increasing LET (Barendsen 1968). The average LET for 6 MV photons is the same as for 4 MeV electrons in regard to the calculated track average or the energy average (ICRU Report No 16 1970). It is in spite of these values quite possible for two different types of radiation, which have the same average LET, to produce different biological effects (Hall 1978). The different kinetics for ciliar activity during photon- and electron-radiation, respectively, must therefore be considered together with some basic physiological facts:

The energy of the beating cilia is provided through the hydrolysis of ATP by the dynein ATP-ase (Gibbons 1965, Gibbons & Rowe 1965). Brokaw & Benedict (1968), furthermore, demonstrated a correlation between the beat frequency and the rate of hydrolysis. Hoffman-Berling (1955) showed a direct relationship between the ATP-concentration and the beat frequency in glycerinated models of trypanosomes and grasshopper spermatozoa. This has also been confirmed by others (Brokaw 1961, 1975, Holwill 1969, Gibbons & Gibbons 1972, Satir 1974).

ATP is presumed to reach the cilium by passive diffusion from the cytoplasmatic matrix, which is continuous with the ciliary matrix (Brokaw 1966).

Investigations using electron microscopy have proved reasonable evidence that the ATP-ase activity is distributed along the length of the cilium (Afzelius 1959, Lansing & Lamy 1961, Gibbons 1963, Sharp et coll. 1979). The rate of ATP-supply by a diffusion process is more than adequate (Brokaw 1966), which means that there is a surplus of ATP available for the ciliary motion under normal conditions. If, then, the ionizing radiation is able to increase the dynein ATP-ase activity, this would result in an increased rate of the ATP-hydrolysis, and, consequently, increased ciliary beat frequency.

Evidence for such a radiation-induced increase of the ATP-ase activity has been found in case of irradiated lymphocytes (Gerber & Altman 1970), but with measurements done after irradiation. As enzyme reactions, however, are very rapid, it is logical to assume that they were present during the irradiation. A stimulation from ionizing radiation has also been demonstrated by Brandt & Baldetorp (1979), who found increased fagocytozing activity of macrophages during irradiation compared to normal circumstances. The radiation must, thus, stimulate the metabolic activity in some way, but there is as yet no clear agreement on its explanation.

Another hypothesis on the stimulation effect is based on the fact that ATP is generated within the cilium, and that more ATP may be created by an increased activity of the phosphotransferase-enzyme adenylate-kinase, as an effect of the irradiation. The presence of adenylate-kinase has been demonstrated in protozoans, as well as in higher species. This enzyme has, for example, been shown to take part in the transphosphorylation on *Polytona* flagella and *Tetrahymena* cilia (Brokaw 1961, Culbertsson 1966). New ATP is thus resynthesized. This reaction is capable of controlling the amount of ATP in cilia and flagella (Saavedra & Renaud 1975, Gibbons & Gibbons 1972), and therefore the concentration of ATP is not solely dependent on passive diffusion.

The effect of irradiation on the physiological events described here, and which for the two radiation qualities used is characterized by the two different curves shown in the Figure, will be considered on the basis of the following postulation: The radiation acts on the same biochemical system in the same manner. The biochemical processes should thus actually represent the same events, but have different kinetics.

The shape of the curves motivates a division of the course of events during three main phases:

1. The initial increase (a in the Figure).
2. The delayed increase (b).
3. The decrease (c).

1. The initial increase

The small but significant increase in the beat frequency is a true

phenomenon previously demonstrated (Baldetorp & Håkansson 1977, Albertsson et coll. 1984) for different photon radiation qualities and different dose rates. Little attention, however, has been paid to this response. Data from these earlier experiments were used in the present study to define the duration of the initial phase, and showed that it lasted 25 seconds when 50 kV X-rays with a dose rate of 0.05 Gy/s were administered, which means following an absorbed dose of 1.25 Gy. Its duration was 65 seconds when ^{60}Co -radiation was administered with a dose rate of 0.017 Gy/s (absorbed dose 1.11 Gy). In the present investigation, its duration was 15 seconds for photons (absorbed dose 1.05 Gy) and 5 seconds for electrons (absorbed dose 0.25 Gy). The increase up to 5% in all experiments may indicate an intervention in the same biochemical system. This system is likely to be an action on the overflow of ATP in the cilium itself, as mentioned earlier (Brokaw 1966). This explanation is, however, only a hypothesis, and requires further investigations for verification. The duration of this initial increase is dependent on the dose rate, but there is a striking difference between photons and electrons which cannot be referred wholly to the dose rate. Electrons obviously appear to have a three-fold greater efficiency, which is difficult to explain, as it does not agree with the general conception of RBE.

2. The delayed increase

Photons and electrons showed differing reactions in this phase. Electrons had a much faster effect, and the peak was reached at a much lower absorbed dose (0.50 Gy) than for photons (1.75 Gy). The biochemical action is not fully understood, but since the amplitude for electrons as well as for photons was about the same, it is assumed that the "amount" of target material is the same in both cases. This question perhaps touches on the "enzyme-release theory" of Bacq & Alexander (1961). It may also be that an activation of the dynein is triggered by the irradiation. Electrons were also more efficient in this phase, and exerted their effect at a third of the dose required by photons. This "Relative-Physiological-Efficiency" (RPE) may be due to the direct ionization action of electrons.

3. The decrease

After a maximal value at the end of phase 2, the beat frequency was already reduced during the irradiation for both photons and electrons. There was, however, even here a difference between the two radiation qualities:

The decrease was much faster for electrons, and the ciliary activity became lower than normal after about 1.5 Gy. For photons, the activity was increased during the whole course of irradiation (10 Gy).

If the "target material" is assumed to be the same at the peak of increase, even here the electrons are much more effective than the photons. It is impossible to give any figure of relative efficiency, and it is not known if the action is on the enzyme kinetics, on the resynthesizing of ATP, or on the passive diffusion of ATP. It is quite probable that all of these factors are involved.

Ogi (1959) further found that after irradiation with 28 Gy electron energy, "the cilia movement decreases without any recovery" (please note that this is not consistent with the figure on page 822 of his article). This statement is very interesting when compared with the results reported by Fujiwara et coll. (1972). After irradiation with 70 Gy of photons these authors found a 30% reduction of the ciliary activity, lasting for at least three hours without any recovery, while Ogi reported a reduction of about 90 per cent during the same time. This indicates at least a three-fold greater efficiency of electrons if the decrease of the ciliary motility and the administered radiation dose required to eliminate recovery of the activity are taken into consideration.

In summary, these experiments together with the studies reported by Ogi (1959) and Fujiwara et coll. (1972), have shown that there is a significant difference between the effects produced by high- and low-energy photons and electrons, and that the conventionally accepted RBE-factor cannot be applied to events during irradiation. A new concept is therefore proposed, called "the relative physiological efficiency". This concept is as yet only considered valid for the present study, which showed the factor: electrons/photons = 3/1. This factor can only be definitely established by further experimental investigations.

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THE EFFECT OF RE-IRRADIATION ON THE TRACHEAL CILIARY CELL ACTIVITY

A Radiobiological Study

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SUMMARY

In order to investigate the radiobiological effect of two irradiations given with a time interval of a few hours, the following experiments have been done: The trachea from twelve rabbits was initially irradiated with 10 Gy in vitro. A second irradiation of another 10 Gy was administered two hours later. The ciliary beat frequency was recorded during the whole procedure. The initial radiation caused a 20 per cent increase of the ciliary activity during the irradiation - a response which was not found during the second irradiation. Between the irradiations, the beat frequency dropped to 20 per cent below the reference level, followed by an 11 per cent overshooting prior to the second irradiation.

Another twelve tracheas served as the control; they were placed untreated in the experimental chamber for two hours, whereupon they were irradiated with 10 Gy. The pattern of the ciliary activity during this irradiation was similar to the findings obtained during the initial exposure of the first experiment. The storage of two hours did not thus affect the conditions of the cilia.

The absence of the great increase of the beat frequency as seen during the first irradiation period may be explained by damages caused by the initial exposure, morphologically seen first a few days later.

Key-words

Trachea; Rabbit; Ciliated cells; Beat frequency; Irradiation; Re-irradiation.

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INTRODUCTION

Earlier experimental work has clearly shown that ionizing radiation changes the rate of the beat frequency in cells provided with cilia (Bohn 1903, Umeda 1927, Frenckner 1939, Ogi 1959, Yamaguchi 1963, Baldetorp et coll. 1976, 1977, Baldetorp and Håkansson 1977, Baldetorp, to be published, and Albertsson et coll. 1984). A 20-25 per cent increase of the beat frequency of the rabbit's trachea was observed during its irradiation, corresponding to an accumulated dose of 1.5 Gy (Baldetorp 1977, Baldetorp, to be published). When using electron-radiation, the frequency increase was found already after a dose of 0.5 Gy, indicating that photons and electrons have differing degrees of effectiveness (Baldetorp, to be published).

The immediate response of the ciliary activity to ionizing radiation was presumed to be due to alterations of the mitochondrial membrane permeability, resulting in a diffusion of a stored pool of adenosine-tri-phosphate (ATP) to the extra-mitochondrial space (Baldetorp 1977) and to an increased enzymatic activity during the ATP-hydrolysis in the cilia (Baldetorp, to be published). In the experimental radiations conducted, a gradual decrease of the beat frequency took place after the maximal increase already during the radiation, and at the end of the exposure, the ciliary activity was only slightly higher than immediately prior to the start of radiation.

Measurements of the ciliary beat frequency immediately after photon-irradiation have shown a depressed ciliary activity with either recovery or a continued depressed activity, depending on the dose administered. Doses of 5 and 30 Gy thus resulted in decreased beat frequency by 5 and 20 per cent, respectively, but recovery occurred within 105 and 135 minutes, respectively (Fujiwara et coll. 1972). Using electron-radiation, Ogi (1959) demonstrated that 0.5 to 14.0 Gy of radiation weakened the ciliary movement, but within three hours the original value had been obtained, so far as the highest dose was concerned.

The complete recovery observed may indicate a repair of the radiation-induced alteration of the ciliary motility system, resulting in a normalized ciliated epithelium, both physiologically and anatomically.

The aim of the present study was to investigate the influence of re-irradiation on the ciliary activity of previously irradiated cells in an attempt to give further knowledge about radiation effects during the early phase.

MATERIAL AND METHODS

The trachea from 24 healthy rabbits was used. The preparation procedure used for the tracheal samples and the technique employed for simultaneous irradiation of the ciliated epithelium and recording of the ciliary beat frequency with a light beam reflex method have been previously described (Baldetorp et coll. 1976) in the literature and are therefore only briefly accounted for in the present paper.

The trachea was placed in an experimental chamber kept at a constant temperature of 37°C, and at a relative humidity of more than 90 per cent, and remained there during a 30 minutes long period of adaptation.

The beat frequency was recorded during 30 seconds immediately prior to irradiation, and the mean value of this recording was taken as the reference level for the individual tracheal sample to be irradiated. The irradiation was performed with a Philip's contact therapy unit (50 kV, 2mA, filter 0.5 mm Al and HVL 0.5 mm Al). The dose rate at the mucous surface was 0.15 Gy per second. Thermoluminescent dosimeters (TLD) were used for the dosimetry.

Twelve tracheal specimens were irradiated with 10 Gy (A) and left in the experimental chamber. Every 15 minutes, the beat frequency was then measured for a period of 30 seconds until re-irradiation (B) took place two hours later. Twelve specimens served as controls and were placed in the chamber for an adaptation period of 30 minutes. Immediately after this period, their beat frequency was recorded continuously during 30 seconds to serve as reference values. During two hours, the registration of the beat frequency was performed every fifteen minutes, as in the first group. The specimens were then irradiated with 10 Gy (C).

The beat frequency was continuously recorded during the irradiation and percentually related for each single second to the reference value obtained

for the individual specimen. The mean values were plotted second-by-second and a smoothed curve was computerized for each exposure.

RESULTS

The mean values of the beat frequency, based on the registrations made after the adaptation period, were for the two groups of specimens 18.5 beats per second ($SD \pm 0.2$) and 19.0 beats per second ($SD \pm 0.2$), respectively.

The results of the different experiments obtained during the first 80 seconds following the start of the irradiations A, B and C are presented in the figure. This figure additionally shows the mean values of the beat frequency change with the standard deviations as plotted every fifteen minutes between the irradiations A and B, and during two hours prior to the irradiation C.

Irradiation A (initial)

As seen in the figure, there was a momentary increase of the ciliary beat frequency by 5.3 per cent ($SD \pm 4.4$) within the first five seconds of exposure. At ten seconds, it increased rapidly to 15.0 per cent ($SD \pm 8.1$) and reached its maximal value of 20.9 per cent ($SD \pm 4.0$) after 15 seconds of irradiation. This peak period was short, and the relative augmentation declined during the following seconds; at 20 seconds of irradiation, the increase was 14.2 per cent of the reference value ($SD \pm 4.2$). The decrease of the ciliary activity continued, though at a slower rate, until the end of the exposure, i.e. after 67 seconds. At 65 seconds, the frequency increase was 3.8 per cent ($SD \pm 2.2$), and at 80 seconds - 13 seconds after completion of the exposure - the frequency was 1.1 per cent above the reference value ($SD \pm 2.1$). There was thus a decided tendency for the frequency increase to be lower than the reference value at the end of the registration.

The beat frequency continued to decrease after the initial irradiation (A) and at 45 minutes after completion of treatment it was 20.1 per cent ($SD \pm 1.1$) below the reference value. Thereafter, an increase took place, which was 11.6 per cent ($SD \pm 1.3$) immediately prior to the re-irradiation (B) as indicated in the table.

Irradiation B (re-irradiation)

Immediately prior to the second exposure, the beat frequency was 20.6 beats per second ($SD \pm 0.1$), or 11.6 per cent ($SD \pm 1.3$) higher than the reference value obtained before the first irradiation, which was 18.5 beats per second ($SD \pm 0.2$). At five seconds after the start of the re-irradiation, the frequency increase was 8.4 per cent ($SD \pm 2.0$). At 15 seconds of irradiation, the ciliary activity further slowed down to 3.8 per cent ($SD \pm 1.7$) above the reference value, and at 30 seconds it was 4.8 per cent ($SD \pm 2.0$). A continued increase of the beat frequency was then seen during the remainder of the irradiation exposure, and at 65 seconds it was 8.8 per cent ($SD \pm 2.9$).

Irradiation C (after two hours)

The mean value of the beat frequency obtained during 30 seconds, two hours prior to the irradiation C, was 19.0 beats per second ($SD \pm 0.2$), a value which was nearly unchanged during two hours, as measured every fifteen minutes. This is shown in the table and in the figure.

The diagram based on the irradiation C, shown in the figure, gives the result of the frequency response to irradiation in the twelve tracheal specimens first treated at 2.0 hours after being adapted to the experimental chamber conditions. The plotted mean values in per cent are the relation between the frequency change during irradiation and the reference value recorded two hours previously.

The pattern of this diagram is seen to be principally similar to that shown in case A. A frequency increase took place during the period 6-12 seconds after the onset of irradiation, followed by a plateau lasting about five seconds, whereupon a slow reduction of the frequency increase was recorded during the rest of the irradiation period.

The frequency change was 1.2 per cent ($SD \pm 2.9$) at five seconds, and it was 8.5 per cent ($SD \pm 7.2$) at ten seconds of irradiation. A maximal increase was reached at 15 seconds (14.6 per cent ($SD \pm 2.0$)). From this time there ensued a decay down to 4.4 per cent ($SD \pm 1.3$) at 30 seconds; at the end of the exposure period, i.e. at 65 seconds, the frequency was 0.6 per cent ($SD \pm 2.1$) below the reference value.

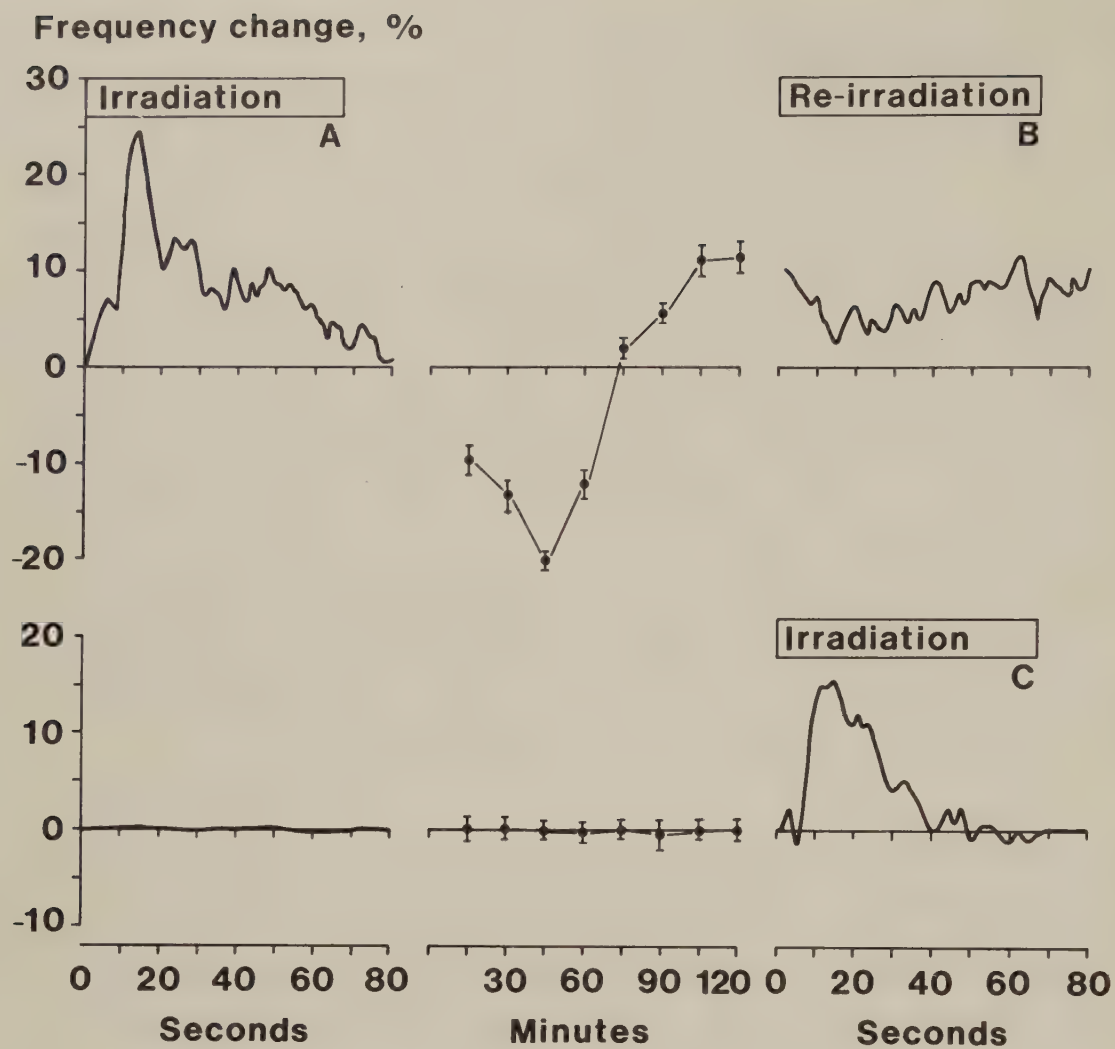


Figure. Changes in beat frequency during the irradiations A, B and C.

⊥: Indicates the mean value with the standard deviation of the frequency change every 15 minutes during a time period of 2 hours.

0% = The reference level.

Table. The mean values of the frequency change in per cent ($\pm$ SD) obtained every fifteen minutes during the two hours between irradiations A and B, and before the irradiation C.

Time (min)	Frequency change ($\pm$ SD)	
	Between irradiations A and B	Before irradiation C
15	-9.7 $\pm$ 1.5	0.5 $\pm$ 0.9
30	-13.3 $\pm$ 1.6	0.2 $\pm$ 1.1
45	-20.1 $\pm$ 1.1	0.0 $\pm$ 1.1
60	-12.1 $\pm$ 1.5	-0.2 $\pm$ 1.3
75	2.0 $\pm$ 1.1	0.2 $\pm$ 1.0
90	5.6 $\pm$ 1.1	-0.5 $\pm$ 1.4
105	11.0 $\pm$ 1.5	0.1 $\pm$ 1.1
120	11.6 $\pm$ 1.3	0.2 $\pm$ 1.2

DISCUSSION

The most important result of the present experiments was the response of the ciliated cells during the re-irradiation phase (B). The peak of the frequency increase that was found during the initial irradiation (A) did not appear. This difference could possibly be explained by the storage of the specimens of trachea in the chamber during the two hours following the initial irradiation (A). Since irradiation performed as initial irradiation (C) after two hours of storage caused exactly the same pattern of response as the irradiation (A), the influence of storage as a contributing factor to the finding during irradiation (B) cannot be asserted. Ogi (1959) and Fujiwara et coll. (1972) showed furthermore that the ciliated epithelium could be kept in the chamber for three hours with unchanged beat frequency.

The present investigation showed the ciliary motility of the non-irradiated ciliary epithelium to be constant, having a beat frequency of 19.0 Hz ($SD \pm 0.2$) during two hours. The frequency can thus be used as an objective and reliable measure of cellular physiological activity for at least two hours.

The effect of irradiation on the ciliary cell activity during the first irradiation period (see figure) is in agreement with the results reported by Baldetorp and Håkansson (1977), indicating that the reproducibility of the same irradiation conditions used in the present study is acceptable. The momentary increase of the beat frequency is supposed to be an increased activity of the enzyme-kinetics in the cilia (Baldetorp, to be published). According to Brokaw (1966) the amount of adenosine triphosphate (ATP) in the cilia is more than adequate for beating. This means that, if the activity of the enzymes involved in the hydrolysis of the ATP increases, then the cilia will beat faster until the reserve of ATP is depleted. The rapid decline of the frequency increase may indicate a reduction of the amount of ATP available as a source of energy for the beating cilia, either due to decreased ATP-diffusion or a reduced production of ATP. A reduced enzymatic activity might also be considered in this connection. This frequency-decrease is even more pronounced at electron irradiation (Baldetorp, to be published). A reduction of ATP-production in other irradiated biological material has been reported from studies with the bioluminescence method (Skog et coll.

1983), and it thus seems logical to assume that the following decrease of the ciliary motility is due to a reduced ATP-production in the mitochondria of the ciliated cells as an influence on the mitochondrial membrane structures due to the irradiation. Even though there were no visible morphological changes in the irradiated mitochondria of preparations for TEM-studies at doses below 30 Gy (Baldetorp et coll. 1977), alterations of the mitochondrial membrane structure caused by smaller doses of irradiation cannot be excluded. It is quite possible that a gradual break-down of the mitochondrial cristae - the ATP-production site - takes place. Although some of these injuries may be repaired during the course of irradiation, the continued exposure increases the number of injuries of the membranes. A further reduction of ATP-production thus occurs, resulting in a continued decrease of the ciliary beat frequency, as is illustrated in case A.

Since both Ogi (1959) and Fujiwara et coll. (1972) found that the cilia were able to beat, although with a slower activity, the ATP-production is probably not completely inhibited. After the lowest value (at 45 minutes after the end of irradiation A in the present study), it began to rise and furthermore surpassed the reference level, overshooting this by 11.6 per cent ($SD \pm 1.3$) at two hours after the irradiation (A). This overshooting was not seen by Fujiwara et coll. (who used a dose rate of 0.34 Gy/s as compared with the actual 0.15 Gy/s in this experiment), but is reported by Ogi (1959), as shown in Figure 2 of his article. The dose rate used by Ogi is not known, but a higher dose rate is supposed to cause even graver injuries that might prevent the frequency from re-exceeding the normal level.

The increased beat frequency registered in the present study immediately prior to the re-irradiation can continue at least three days after a single dose of 10 Gy, as was shown by Albertsson et coll. (1984), who irradiated the rabbit trachea in vivo and measured the mucociliary wave frequency in vitro on ten consecutive days after treatment.

The re-irradiation started at a beat frequency level of 11.6 per cent above the initial value. During the first 15 seconds, a slow retardation took place, instead of the increase seen in case A. The activity then slowly returned to the same level as before exposure. The findings may indicate that the first irradiation had caused changes in the motility system that

lasted for more than two hours.

Albertsson et coll. (1984) reported apparent morphological alterations of the irradiated ciliated epithelium first 4-8 days after the treatment. It is therefore quite possible that the lack of frequency increase during the re-irradiation is due to radiation damages made by the first exposure, but detectable with electron microscopy technique first a few days later. These injuries do not appear to seriously affect the ciliary motility mechanism or the ATP-production during these days.

The delay of 2.5 hours between the sample preparation and the irradiation obviously did not seem to influence the response of the ciliated cells. The same pattern of ciliary activity during irradiation was recorded (see the figure, A and C).

It may also be possible that the increased beat frequency measured prior to the re-irradiation and during 1-3 days after the single dose irradiation depends on viscoelastic alterations of the mucous. The amount of mucous secretion may influence the ciliary activity, as well. Further investigations are needed to clarify these questions.

In conclusion, the lack of frequency increase during the re-irradiation can probably be explained by radiation damages caused by the initial irradiation. These damages do not seem to affect the ciliary motility system drastically, since the cilia continue to beat during the first few days after exposure to 10 Gy of irradiation, as shown by Albertsson et coll. (1984).

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THE EFFECTS OF 10 Gy SINGLE-DOSE IRRADIATION ON THE CILIATED EPITHELIUM
MEASURED DURING AND ONE-TO-TEN DAYS FOLLOWING IRRADIATION.
A Comparative Physiological and Morphological Study.

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Abstract

Experiments recently done in this laboratory have shown that ionizing irradiation can result in an increase in the ciliary beat frequency of the trachea *in vitro* even during ongoing irradiation, implying an immediate stimulation of the physiological activity of the cells. This phenomenon is not fully understood and is by itself contradictory to general radiobiological concepts. Ultrastructural investigations on specimens taken immediately after irradiation (10 Gy) have, so far, not shown any changes. It has therefore seemed useful to extend the investigations by examining the effects of *in vivo*-irradiation (10 Gy) on rabbit trachea during the first 10 days after treatment. The following results have been obtained:

Phase I: On days 1 - 3 after irradiation the beat frequency showed a slight increase (10 %) within the irradiated part in comparison with a non-irradiated area of the trachea. No pronounced ultrastructural changes were found these days.

Phase II: On days 4 - 7 after irradiation the mucociliary activity was reduced in those places where it could be measured. The surface of the cilia assumed a generally destructed appearance with elongated cilia and disorganisation. TEM-pictures showed an increased amount of goblet cells and signs of membrane damage such as cytoplasmic extrusions at the apical end of the cells.

Phase III: On days 8 - 10 after irradiation a normalization of the tissue gradually took place with a beat frequency returning to normal. The ciliary surface showed a recovery process which also could be observed in the TEM-pictures.

KEY WORDS: irradiation, tracheal epithelium cilia, immediate effects, late effects, physiological activity, scanning electron microscopy, transmission electron microscopy.

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Introduction

The manifestation of the biological effects of ionizing irradiation is time-dependent, as has been demonstrated in both physiological and morphological studies of these effects. Earlier investigations would seem to indicate that modern methods detect the physiological effects earlier and with greater facility than is the case when the morphological effects are studied. Baldetorp et al (1976) and Baldetorp (1977) thus found an increase of the mucociliary wave-frequency in the tracheal ciliated cells of the rabbit coming as a stimulatory effect during irradiation by photons at different dose rates. This effect was usually detectable at an accumulated dose of 1.5 Gy, but was not accompanied by any morphological changes which could be seen with electron microscopy.

Ogi (1959) studied the effects of beta-irradiation on the ciliated cells after completion of irradiation. He found a decrease of the beat frequency, but also noted recovery after 2 - 3 hours in the dose range of 7 - 14 Gy.

Fujiwara et al (1972) found principally the same effects after irradiation with photons. Morphological studies were, however, not performed. The asserted stimulatory effect of irradiation is still a matter of dispute, and the experiments reported in this paper have been conducted in order to further investigate the seemingly contradictory findings of others, as described above, in the hope of finding more information, and in order to compare the immediate and later effects of irradiation.

In our study, the trachea of the rabbit received 10 Gy *in vitro*, and the beat-frequency was measured during this irradiation. Electron microscopy was made on preparations immediately following irradiation. The results have been compared with those obtained from experiments where the trachea of the rabbit was irradiated with 10 Gy and measurements corresponding to those of the present study

made during ten consecutive days after irradiation.

Material and Methods

"Immediate Effects": Irradiation in vitro

Forty rabbits were used for the examinations. They were sacrificed by a blow on the skull in order to avoid side-effects, whereupon 70 mm of the trachea was extirpated and divided in two equal parts. They were then stretched to about 20 per cent more than the contracted state, and placed in the experimental chamber. One specimen was placed in a lead-shielded box, and the other mounted for measuring the beat-frequency. The details have been described earlier (Håkansson and Toremalm, 1965; Baldetorp et al. 1976), since the well-known light-beam reflex recording was used. Ten Gy were given to the specimen, and immediately after the irradiation, sections were cut both from the irradiated trachea and the control in the lead-shielded box, which were studied with Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). In order to obtain energy quantities which could be used for comparison with the "late effects" (1 to 10 days after treatment), the *in vitro*-irradiation was performed with two different qualities:

1. X-ray, generated at 50 kV, 2 mA, filter: 0.5 mm Al, Half Value Layer: 0.5 mm Al, focus-object distance: 60 mm. The dose rate was 0.05 Gy/s. A Philips contact therapy apparatus was used. Each tracheal specimen received 10 Gy. Twenty rabbits were used.
2. Gamma-radiation from ^{60}Co , source diameter: 15 mm, source to front of diaphragm distance: 300 mm, source-object distance: 610 mm and field size: 130 mm x 130 mm at 610 mm source-distance. The dose-rate was 0.017 Gy/s. A Siemens Gammatron III unit with multiplane collimator was used. Twenty rabbits were used.

Dosimetry-measurements were carried out with thermoluminescent dosimeters (TLD) at the place in the experimental chamber where the centre of the tracheal mucous membrane was lying during exposition.

"Late Effects": Irradiation in vivo

Ten rabbits were used and the examination was performed as described above. The first measurements were made one day after irradiation on one of the rabbits, and the others were investigated on the following consecutive days. The last rabbit was examined on day 10 after irradiation.

Irradiation was performed by a conventional Siemens X-ray unit using 160 kV and with a HVL: 4 mm Al. Skin-Source Distance (SSD): 50 cm. Dose-rate = 0.013

Gy/s. Before the irradiation, the animals had been rendered unconscious by intraperitoneal injection of pentobarbital (40 mg/kg body-weight). The rabbits were laid on their back, and the upper part of the ciliated trachea (20 mm) was irradiated. The caudal limit of the treated area was lead-shielded, and the direction of the beam was arranged in a cranial angle, in order to strike the caudal part along a cartilage arc. The spatial distribution between the irradiated part and the control part was 40 mm. The two sections were placed in the experimental chamber as above, and the beat-frequency was recorded from each preparation. Immediately prior to measurement, sections were cut for SEM- and TEM-examinations.

Preparation for SEM

The pieces for SEM-examination were not rinsed, but were placed directly in 2.5 % glutaraldehyde for fixation during 12 hours (in 0.15 M cacodylate buffer). The pH of the solution was 7.3. They were then transferred into the same buffer, and were later osmium-fixed in 1 % osmium tetroxide in 0.15 M cacodylate buffer for two hours.

Dehydration took place with graded series of ethanol, after which the preparations were transferred to Freon IF 618. The specimens were later dried according to the critical point method in Balzer's 000 Critical Point Dryer. They were finally sputter-coated with gold plus palladium in a Polaron SEM-coating unit E 5000. Then they were examined in a Cambridge Stereoscan Mark II A or a Zeiss Nanolab Electron Microscope. The microscopes were operated at 20 kV.

Preparation for TEM

The samples were fixed as for the SEM-preparations and also in 1 % OsO_4 in phosphate buffer (pH 7.4) for two hours, rinsed in 0.15 M cacodylate buffer, dehydrated in alcohol and embedded in Vestopal W or Epon. Sections of 1 μm thickness were cut out on an LKB-Ultratome, stained with toluidine blue and examined in a light microscope. Ultrathin sections were cut out and contrasted with lead citrate and uranyl acetate, or *en bloc* with 0.5 % uranyl acetate. A Zeiss EM 10 Electron Microscope was used.

Statistical Analysis

Statistical analyses were done with regression analysis using the package "Minitab".

Results

Immediate Effects

In the normal trachea, the beat-frequency was 987 ± 30 beats/min. In those specimens irradiated with 50 kV X-rays, the frequency increased up to about 25 % at an accumulated dose of 1.5 Gy (Fig. 1, partly extracted from Baldetorp, (1977)).

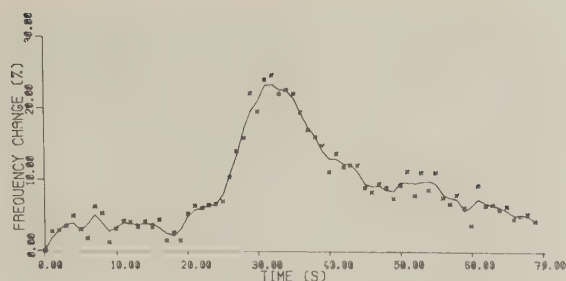


Figure 1. The change in the beat-frequency during irradiation. 50 kV, dose rate: 0.05 Gy/s. Each point represents the mean value for 20 rabbits.

It then decreased to a level just above the normal value after a dose of 3.0 Gy. ^{60}Co -irradiation caused a corresponding increase (Fig.2) up to 22 % following an absorbed dose of 1.3 Gy. The increment lasted longer (30 s), and declined very slowly.

Morphology: Specimens taken immediately after the irradiation were investigated by SEM and TEM. No deviations from the non-irradiated part (cf description below) were seen.

Late Effects

The beat frequency of the irradiated part could be measured over the whole surface the first days after irradiation. When measured second-by-second, it varied within a range of 4 Hz as has generally been found in earlier experiments. Figure 3 shows the mean value in beats per minute from each rabbit from day 1 to day 10 after irradiation, related to the mean value as measured in the non-irradiated area. It is noticed that, especially during the first days, the quotient has a higher value than 1.0, which would indicate a stimulation process.

It was, however noticed that, during days 3 to 8, only part of the mucous membrane could be used for determining the frequency; the surface seemed inactive, and reached its lowest point on days 4 to 6, when only separate small areas were active. India ink was placed on the surface, and it could then be established

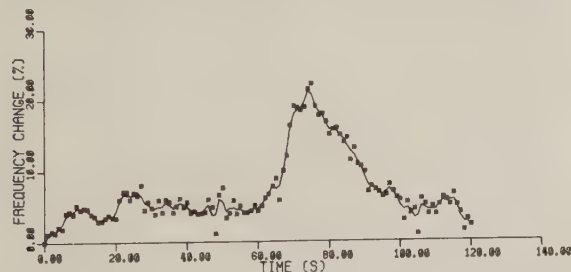


Figure 2. The change in beat-frequency during irradiation. ^{60}Co , dose rate 0.017 Gy/s. Each point represents the mean value for 20 rabbits.

Beat frequency quotient, T_1/T_2

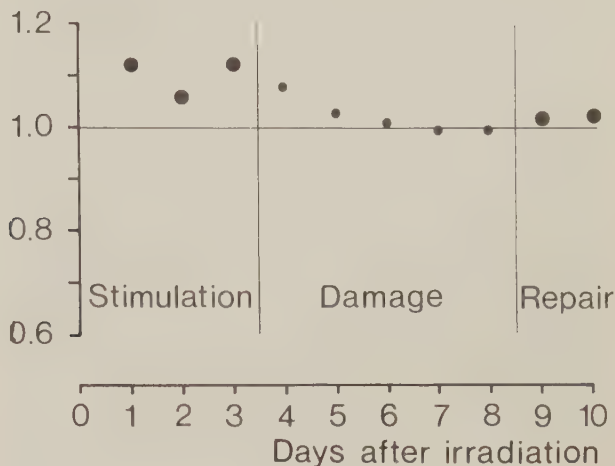


Figure 3. The ciliary beat-frequency within the irradiated part of the trachea (T_1) in relation to the beat-frequency in the non-irradiated part of the trachea (T_2). One point is the experimental data from one individual rabbit, and the ten results were obtained from the first day after irradiation to day 10. ● = normal, ○ = abnormal surface.

that, on certain small spots, the particles moved only in circles, while as a whole the mucous carpet was not moved as in the normal cases. The frequencies of the active cilia were, however, recorded, and the results are shown in Fig.3, where the large points represent recording from a surface with normal transport capacity and the small points are recording from a surface with reduced transport capacity.

The amplitude of the oscillations from these cilia groups was, furthermore, lower, as compared with those recorded on the non-irradiated trachea, or those recorded one or two days after irradiation. This may indicate a reduced ciliary efficiency in regard to their strokes. It is nevertheless interesting that some groups of cilia showed a frequency slightly above, or similar to, that recorded (in the same way) from the non-irradiated part. The curve is interesting *per se*, even if it does not reflect the true mucociliary activity of the mucous membrane. This condition is more clearly visualized in the SEM- and TEM-recordings.

Morphology: (Scanning Electron Microscopy). In the non-irradiated material, a smooth carpet of cilia with an even structure and only a few blebs were seen, as well as a small number of goblet cells coming up to the surface with a coating

of small microvilli. In the irradiated material, changes were noted from day 1 to day 10. To facilitate summing up the findings, it seemed preferable to use a scoring system, where the different pictures were judged by four persons, independently of each other. This system employed units from 0.0 to 4.0, the last figure describing the figure with the greatest amount of injury. To obtain a more gliding scale, and perhaps a more refined judgement, the 0.5-fraction was put between the numbers.

The Score System

The following definitions were worked out for the respective values used:
0.0 (Fig. 4 a,b): Normal surface with clean cilia having a minimum of blebs (5 - 24 on a defined area), and with regular arrangement. In rare cases, with visible goblet cells having intact microvilli. No mucus granula or fibrin.

1.0 (Fig. 5 a,b): The cilia have a regular pattern which is constant, as can be seen on a 1000 x picture. Blebs occur especially on the tips of the cilia, but also on the sides (to a number of 25 - 49 on a defined area at 5000 x magnification. Only solitary mucus granula are to be found on the surface. The goblet cells have normal microvilli.

2.0 (Fig. 6 a,b): Blebs to a number of 50 to 74 on a defined area at 5000 x magnification. A rich amount of goblet cells with their content protruding on the surface having disintegrated and shortened microvilli. Some of the goblet cells have emptied their contents and mucus granula are seen on the surface. Many cilia are seen adhering to one another.

3.0 (Fig. 7 a,b): Blebs occur to a number of 75 - 99 on a defined area at 5000 x magnification. The cilia are arranged in a regular pattern. Thick masses of emptied goblet cells with mucus granula are spread over the surface, the remaining goblet cells having almost no microvilli. There is a coating of detritus.

4.0 (Fig. 8 a,b): Blebs are found to a number of 100 or more on a defined area at 5000 x magnification. The cilia are in a disorderly pattern, and have disintegrated. The goblet cells are emptied. The surface is covered with detritus and probably with fibrin. A few cilia with intact structure can be seen here and there. Figure 9 shows the results of the evaluation by this method.

Each point represents the mean value of the judgement of the four persons. The curve was statistically analyzed, and the results showed a significant difference between the appearance of the surface during the first three days and those between days 4 and 7. Day 8 and the follow-

Figure 4a. Score 0.0. Normal, ciliated trachea.

Figure 4b. Enlargement from Fig. 4a. Note the clean cilia.

Figure 5a. Score 1.0. The cilia appear normal. At the arrow a goblet cell is emptying its content with normal microvilli on the surface.

Figure 5b. Score 1.0. Enlargement from Fig. 5a.

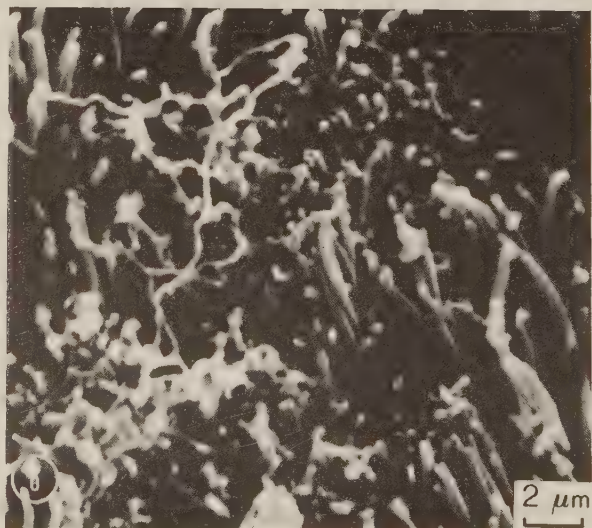
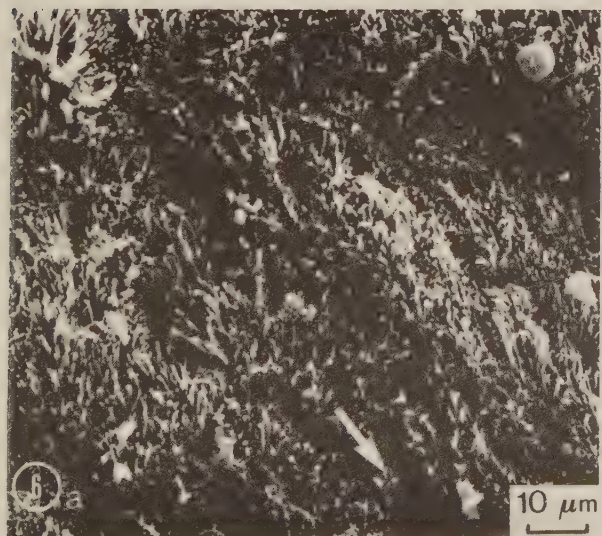
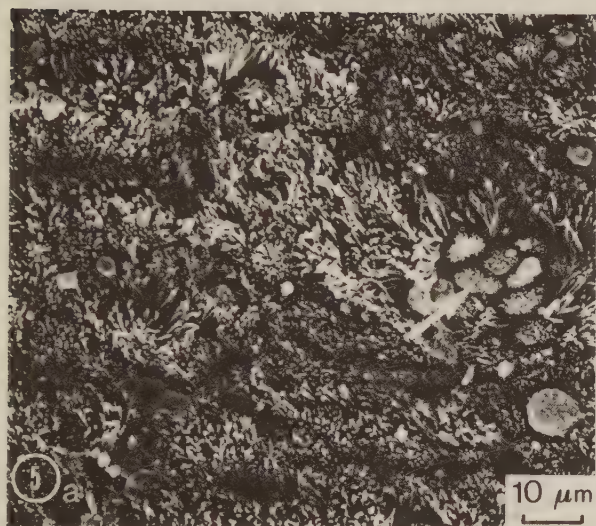
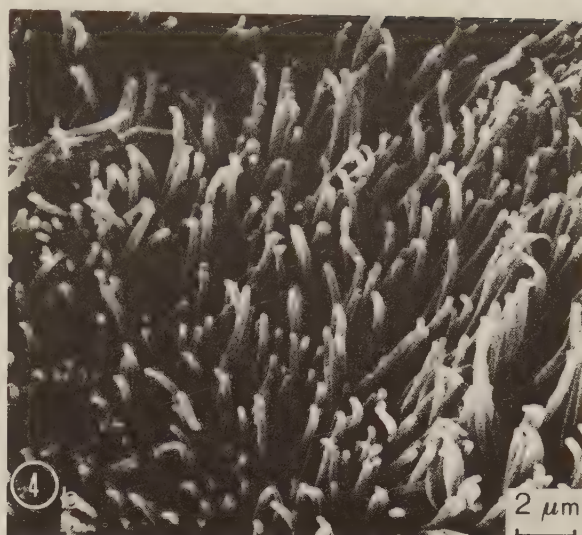
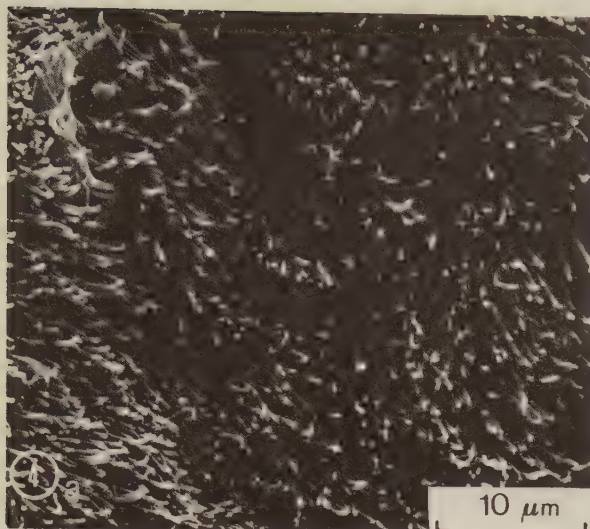
Figure 6a. Score 2.0. At arrow: shortened microvilli on the contents of the goblet cell.

Figure 6b. Score 2.0. Enlargement from Fig. 6a. Rich amount of detritus.

ing days also differed from the values of days 4 - 7 ($p < 0.002$). The variation as a function of time seemed to be both linear and quadratic. In order to see if different structures followed the general impression of the consecutive pictures, an attempt to apply more objective measurements was made by measuring the thickness of the cilia on the pictures with 5000X magnification. This was also done by four persons independently of each other. Thirty cilia from each specimen were calculated. Bearing in mind that the diameter of the cilia varies considerably at the tips, measurements were only made where there was even thickness. In SEM, the diameter of the cilia was in the range of 0.3 - 0.4 μm , and therefore not consistent with the accepted value. The SEM-measurements are only relative and the contribution of the gold-palladium film is included. The variation of the ciliary diameter in relation to time was, however, statistically significant ($p < 0.002$), with both a linear and quadratic effect (Fig. 10). The exact value was then measured on TEM, which showed the same pattern (Fig. 10). Measurements from days 1, 5 and 10 are presented. The diameter is here within the accepted values of 0.2 - 0.3 μm , with the lowest value on day 5 after irradiation.

Transmission Electron Microscopy, (Non-irradiated material): The normal trachea showed the characteristic layer of basal cells, ciliated cells and goblet cells above a supporting structure of collagen fibres. The cilia had the normal length of about 7 μm , and a thickness of 0.2 - 0.3 μm . Rich amount of microvilli from the

The effects of 10 Gy single-dose irradiation



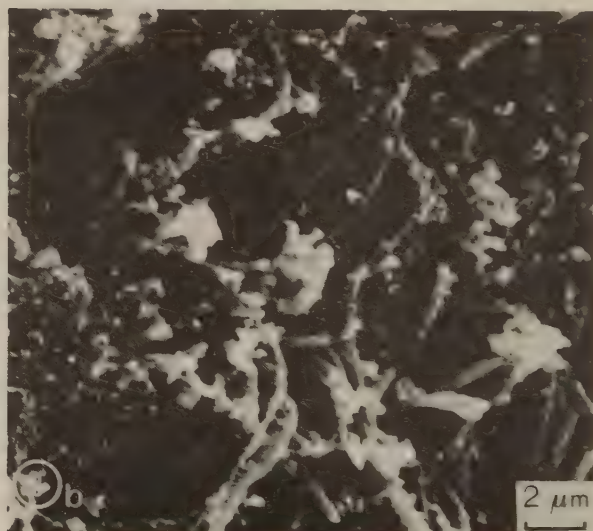
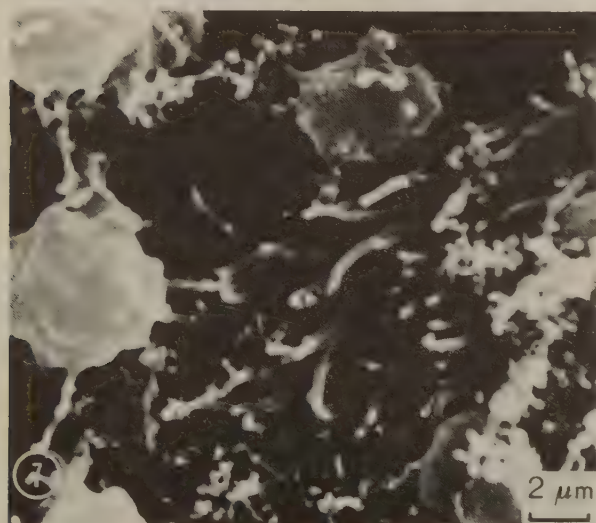
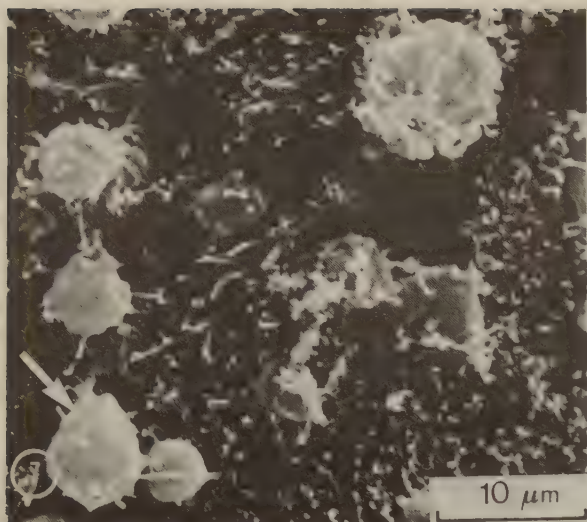


Figure 7a. Score 3.0. Granula from emptied goblet cells (arrow). Shortened microvilli.

Figure 7b. Enlargement from Fig. 7a.

Figure 8a. Score 4.0. Heavily injured surface.

Figure 8b. Enlargement from Fig. 8a.

surface of the ciliated cells were found between the cilia. They had a length of 3 - 4 μm . Inside the ciliated cell, the nucleus was in the basal part. The usual configuration of the nucleus was oval or circular, with no invaginations. Endoplasmatic reticulum with the usual smooth appearance was found, as well as free ribosomes. The goblet cells between the ciliated cells had vesicles with mucus granula. Some of the cells had come to the surface of the epithelium layer, where small microvilli were seen on the membrane. These microvilli had a length of 0.4 - 1.0 μm . Some of the goblet cells had emptied their contents, as apocrine cells and only remnants of the cell bodies were interspaced between the ciliated cells. Mitochondria were seen both in the

basal cells, the goblet cells and in the ciliated cells. They had their usual appearance, being of oval or elongated form, with unbroken cristae.

Irradiated material: On the basis of the findings with the score system, and the results from measuring the diameters of the cilia, it seemed preferable to look at the TEM-pictures as representing three time periods after irradiation with 10 Gy: Phase I; days 1 - 3 (called the stimulation phase), Phase II; days 4 - 7 (the damage phase), and after day 8 Phase III (the repair phase).

Phase I (Stimulation): Many of the cilia gave an impression of having a greater distance between the microtubules and the outer membrane. The 9+2 structure was clearly seen, and a small bleb was found

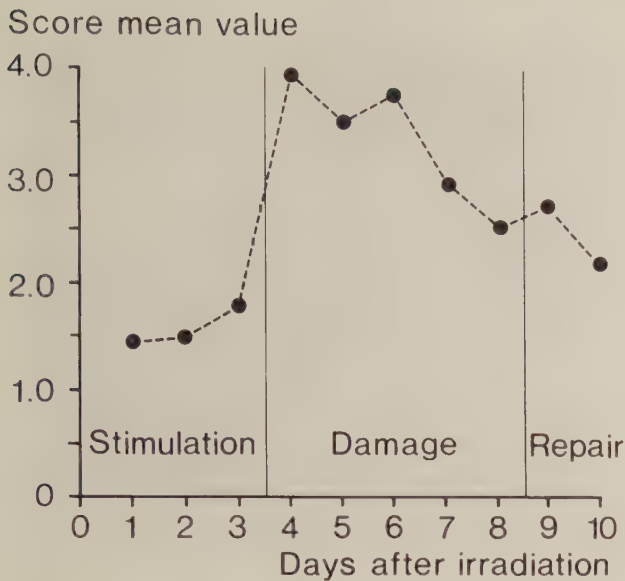


Figure 9. Mean value for the scoring of the SEM-pictures.

at the tips of some cilia. The mitochondria were swollen, and some of them had broken cristae. The form of the nuclei did not have its usual even, rounded or oval structure. In some cells, a ductal invagination or lobulation of the nucleus was noted. The chromatin was distributed within the whole nucleus, but with a tendency to accumulate at the membrane where the pores of nuclei could be detected. The goblet cells occurred more frequently than is normal, and showed a distinct and perceptible endoplasmatic reticulum.

Phase II (Damage): The cilia were not swollen as in the first phase, but had blebs on their sides. The 9+2 pattern seemed unchanged. The mitochondria were still swollen, and broken cristae could be seen. There was also a tendency to an accumulation beneath the basal bodies of the ciliated cells. The amount of goblet cells were even more increased. From the apical part of some ciliated cells, cytoplasmic evaginations or protrusions contained amorphous material and it was difficult to observe any distinct membrane (section effect?), but it could be imagined on certain small spots (Fig. 11). Endoplasmatic reticulum and Golgi complex were clearly seen, especially in the goblet cells, which were found to have large mucous vesicles with small granula (Fig. 12). The chromatin was distributed as described earlier, but small areas with less density were seen. The nucleolus were clearly visualized (Fig. 13a). The nucleus membrane was often lobulated and showed a rich amount of nuclear pores (Fig. 13b). During this second phase, many heterophagic vacuoles were seen.

Phase III (Repair): The cilia had an even membrane, and cross-cut sections showed

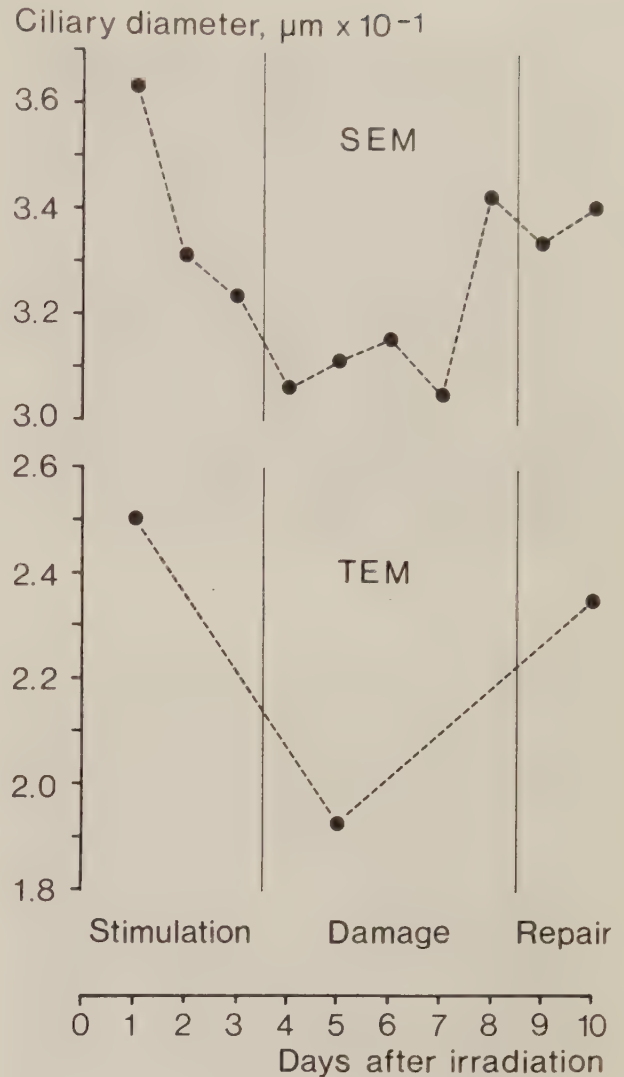


Figure 10. Upper picture: Diameter of the cilia as measured from the SEM-pictures. Each point represents the mean value of 30 measurements. Lower picture: Diameter of the cilia measured from TEM-pictures.

the usual 9+2 pattern. The distance between the microtubules and the outer membrane was as in the control material. The mitochondria also showed a distribution similar to that of the controls. During the early stage of this phase, some nuclei had a lobulated form and showed some areas with less density. The distribution of chromatin had not changed. Ribosomes and endoplasmatic reticulum were clearly seen in the cytoplasm. The number of goblet cells was likewise increased, and heterophagic vacuoles were also seen. As a whole, these phases overlap each other, which indicates dynamic processes that take part at different sites in the ciliated cells.

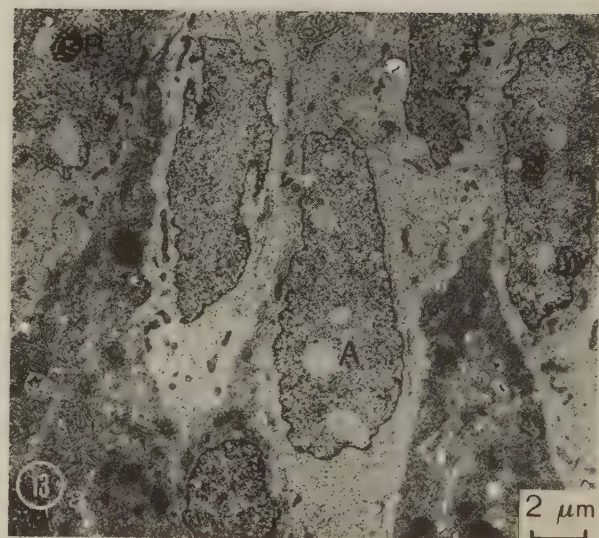
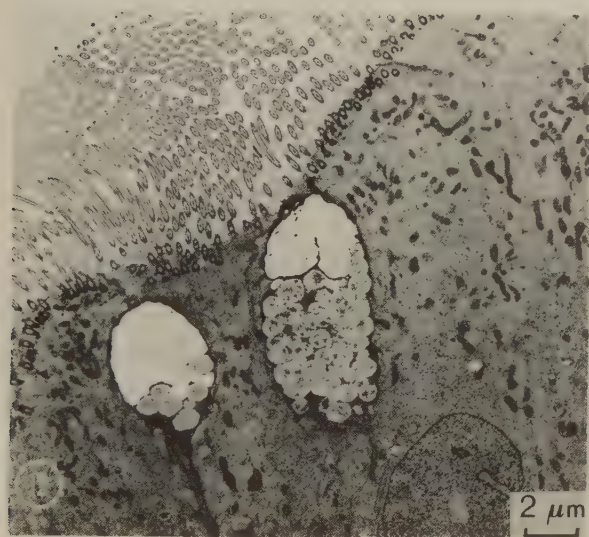
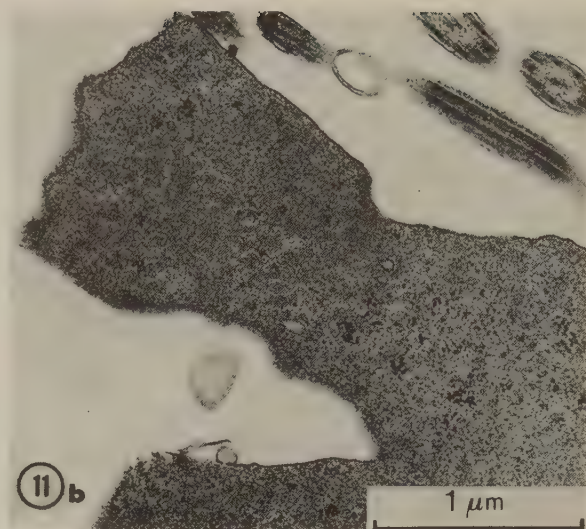
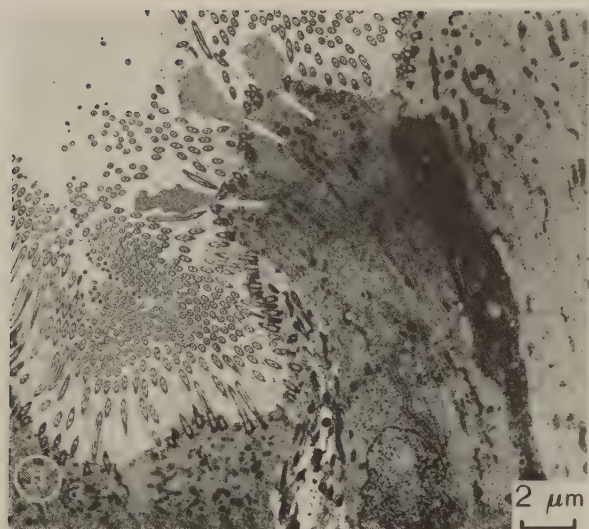
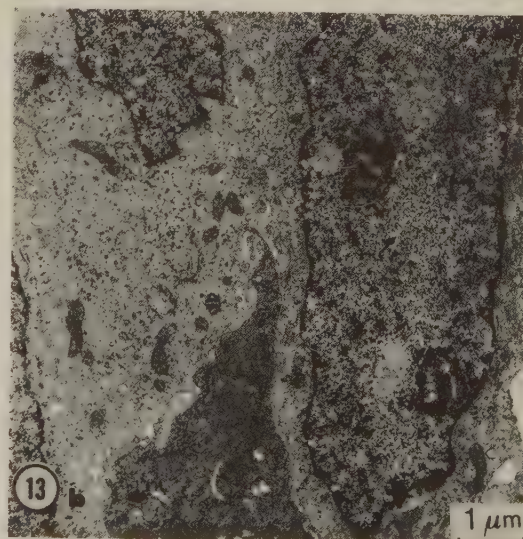


Figure 11a. Arrows: Cytoplasm protruding from the ciliated cell.

Figure 11b. Higher magnification of the extrusions seen in Fig. 11a. Note the existence of a membrane on the right side of the protrusion.

Figure 12. TEM-picture of the irradiated ciliary epithelium. Note the large mucous vesicles in the goblet cells.

Figure 13a.b. TEM-pictures. Note the multilobulated nuclei, the zones within the nucleus with low density (A), and the marked nucleoles-(B).



Discussion

In order to be able to accurately compare the "immediate" and "late" (1 - 10 days after treatment) effects of irradiation, it is necessary to first define these concepts. Immediate effects are here considered to be those which can be measured during irradiation, while late effects are those which are detectable one-to-ten days following irradiation. Concerning the immediate effects, they are induced by a radiation energy varying from low X-ray irradiation at 50 kV to high-energetic irradiation using 1.25 MeV photon irradiation. The Compton effect can be assumed to dominate at both of these levels of energy, as well as that energy used in the late-effect experiments, i.e. X-ray irradiation at 160 kV. The trials are thus acceptable from the standpoint of irradiative energy. The differences in dose rate can also be proved: 0.05 Gy/s was used in the attempts with 50 kV irradiation, and 0.017 Gy/s was utilized in the trials with ^{60}Co . When 160 kV X-rays were used, there was a dose rate of 0.013 Gy/s.

Immediate effects

The increase in frequency seen during irradiation is rather confusing and quite difficult to explain. This initial effect is nonetheless indisputable. It is also confirmed in experiments with lower ciliated organisms. As a matter of fact, it was proposed as early as 1903 by Bohn. The general assumption today is that adenosine triphosphate (ATP) serves as energy for the ciliary motility, and that the concentration determines the beat frequency (Satir, 1974). According to the "enzyme release theory" proposed by Bacq and Alexander (1961) as an effect caused by irradiation, the increased speed of ciliary movement may be explained by a release of ATPase, and the immediate effect will thus take place within the cilium itself. This increase is not followed by a change of the electrophysiological activity (Baldetorp et al. 1983). Immediately after the irradiation, both Ogi (1959) and Fujiwara et al (1972) found that the beat-frequency decreases. Although Ogi used beta-particles and Fujiwara et al, photons, their results were principally the same: a decrease followed by a return to a normal value within a period of 2 - 3 hours (dose range: 5 - 30 Gy).

The decrease of activity during the late phase of irradiation may be explained by a reduced pool of ATP in the cilia and a lack of newly synthesized ATP from the mitochondria. It is known that a resynthesis of ATP can take place within the cilia from available nucleosides, by means of the action of phosphotransferase on adenosine diphosphate (ADP) (Saavedra and

Renaud, 1975). Nothing is known, however, about the length of the time-factor of this resynthesis.

No morphological changes could be observed on the preparations taken immediately after irradiation. Ogi (1959) found no such changes, either, in this dose-range. His findings in this respect were confirmed by Baldetorp (1977), who found that it requires doses above 30 Gy to produce changes detectable with electron-microscopy.

Late effects

Phase I (Stimulation): Recordings of the beat-frequency showed that it was higher than normal during days 1 - 3 following irradiation. This is interpreted as indicating that the beat-frequency is stimulated by irradiation. It is, however, not logical that this phenomenon can be explained in the same way as the stimulation observed during irradiation. ATP is delivered by the mitochondria, and if a true stimulation exists, caused by irradiation, an increased synthesis of ATP may take place, thus increasing ciliary activity, as measured during days 1 - 3.

Another possibility is that there exists a feed-back mechanism from the cilia to the mitochondria, and that if irradiation has caused depletion of the ATP, this gives a signal to the mitochondria to synthesize more ATP.

It is interesting to note the difference between the rates of increase of the activity found in the studies of the early effects and those found in the studies of the late effects. There is 20 - 25% increase of activity during irradiation, but the increase is only 10% during days 1 - 3. TEM-examination of the irradiated *in vivo* cells reveals that they contain mitochondria with swollen membranes, which indicates that they are affected by irradiation. It is at present not possible to say if this stimulation seen on days 1 - 3 is due to damage of the membrane, with an eventual leakage of ATP. During the first three days, there are no pronounced ultrastructural changes. The conclusion must be drawn that these first three days are a phase of stimulation, although the background for this is not known.

Phase II (Damage): This first phase passes into a phase where the mucociliary activity is reduced in those places where it can be measured. At the same time, the surface of the cilia assumes a generally destructured appearance, with elongated cilia and disorganisation. In the later part of this phase, most of the ciliary cells have been put out of action, corresponding to the appearance of the ciliary surface seen by SEM. The later effects of irradiation are better evaluated by SEM-study than by TEM-examination.

The second phase, i.e. days 4 - 7, after irradiation reveals damage to the structure: there is a disintegration on the surface, and the diameter of the cilia is diminished. The observed failure of the mucous carpet to move can also be explained by mechanical action on the ciliary crown (Dirksen and Satir, 1972). This can be assumed to be indicated by the findings of the increased numbers of goblet cells, which is most dominating on days 4 - 7 following irradiation. If these cells empty their contents, the carpet covering the ciliary cells ought to be of higher viscosity.

The most likely explanation is that both stimulating and damaging effects interact during this period.

Phase III (Repair): During this period, a normalization of the tissue gradually took place, perhaps because the trachea had been in the host for up to 10 days, and the prerequisites for the above-mentioned assumption are then most favourable. It is also consistent with the increase in diameter of the cilia, and a scoring of lower values when judging the SEM-pictures. The beat-frequency tends to be the same as in the nonirradiated area of the tracheal mucous membrane. The TEM-pictures also show this normalization process.

The third phase may therefore be regarded as the "period of main repair". This does not exclude, of course, the possibility that reparative processes begin to work at a much earlier phase. The role of blebs on the cilia is difficult to explain. Their origin may be spots of weakness of the membrane of the cilium (Dalen, 1983).

Conclusion

Finally our present knowledge of the effects of 10 Gy irradiation given to the tracheal epithelium can be summarized in figure (14).

Acknowledgements

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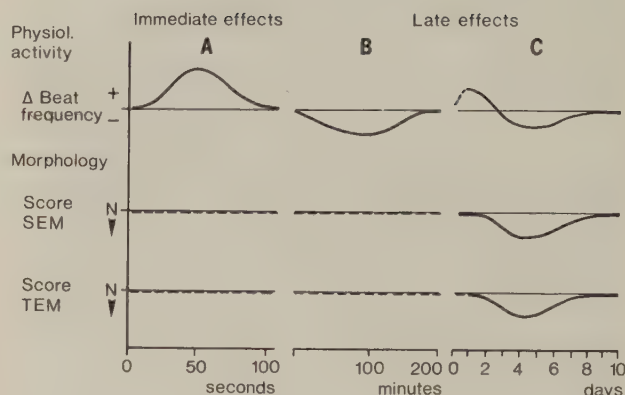


Figure 14. A summary of the events of the ciliated tracheal epithelium during and after irradiation with 10 Gy.

A: Baldetorp (1977) and Baldetorp (this paper).

B: Ogi (1959); Fujiwara et al (1972).

C: Albertsson (this paper).

+, - = Higher or lower activity, respectively, than normal.

N = Normal structure.

▼ = Deviations from the normal structure in arbitrary units.

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Discussion with Reviewers

B. Afzelius: Tracheal cilia are mucous-propelling ones and treatments that give an increased beat frequency may not necessarily be due to effects on the cilia themselves. The primary effect may be on the amount and viscosity of mucous. Have these factors been checked?

Authors: The amount of mucous has not been checked but we have tried to measure the viscosity or the tenacity, because the mucous is not a Newtonian liquid. Commercial apparatus cannot be obtained because it is not possible to get more than a few µl of mucous. We have therefore developed an apparatus for the purpose: Håkansson and Toremalm: A method for determining the viscoelasticity tracheobronchial secretions. Am. Rev. Resp. Dis. 1970. 102, 1, 42-47. The investigations have not shown a change in the viscoelasticity due to irradiation.

B. Afzelius: Increased frequency occurs at higher temperature, e.g. at fever. Did the animals have fever after irradiation?

Authors: No, and this is because the size of the irradiation field is small (10 x 20 mm). We have measured the temperature beneath the trachea and there is an increase in the temperature of 0.8°C at its maximum. This corresponds to an increase of the beat frequency of less than 25 beats/min.

B. Afzelius: There is a subgroup of the immotile-cilia syndrome in which the cilia have a hyperfrequent beat (>13 Hz) and which by Pedersen et al. Europ. J. Resp. Dis. 64 Suppl 127, 78-90, 1983 is called primary ciliary dyskinesia.

Although the cilia in this subset of the syndrome beat rapidly the beat is inefficient. Do the authors have any information on whether the cilia in the "stimulatory phase" of their irradiation experiment do useful work? If not, the term "stimulation phase" may be misleading.

Authors: When Pedersen et al. find ciliary dyskinesia they claim that the recording of the beats gives irregular wave pattern. In our case during the stimulatory phase the waves were quite regular.

B. Afzelius: On days 4 - 7 post-irradiation ciliary transport goes in circles rather than straight as it should for useful work. How about the orientation in the stimulation phase?

Orientation can be evaluated in the living state and on ultrathin sections and also by noting the orientation of the ciliary tips in the SEM. Ciliary tip is bent in the same direction as the effective stroke (Veerman et al. Pediatrics 65, 698-702, 1980).

Authors: During the "stimulation phase" the india ink flowed cranially with the mucous. Unfortunately we have not looked upon the orientation on the sections. We will do this.

B. Afzelius: How do the authors visualize cilia that can beat with the dynein detached from the microtubules? If this is the idea of the "enzyme release theory" then the current concepts of ciliary motility have to be revised.

H. Dalen: The authors have suggested that the changes in the ciliary beat-frequency following irradiation may be coupled with the release of dynein. If so, have any morphological alterations of the dynein arms been observed in the present study?

Authors: We did not observe any morphological alterations in the dynein arms of the cilia. We think that the irradiation increases the access to ATP. In what way we do not know. According to Brokaw (Mechanics and Energetics of cilia, Am. Rev. Resp. Dis. 1966. 93 No 3 part 2) the supply of ATP in short cilia "will be more than adequate". Therefore we think that the irradiation is capable of giving more energy to the system, at least during the first seconds of treatment.

B. Afzelius: Resynthesis of ATP by the $2ADP \rightarrow ATP + AMP$ reaction will not suffice for keeping an ATP-requiring process going for long, as the store of ATP always is quite small due to its relative toxicity. Any comments?

Authors: We cannot refute your statement and are not able to answer your question.

K.E. Carr: Can any figures be given of the changes in amplitude associated with ciliary beat?

K.E. Carr: Were there any changes in the basal lamina of the epithelium?

Authors: Probably yes. Ten Gy, however, seems to be a border line between detectable and undetectable changes. We want to come back to your question when we have done various single-dose experiments.

R.F. de Estable-Puig: Why were different irradiation protocols utilized for the in vitro and in vivo experiments?

Authors: The experiments where a stimulation effect was noted were made in vitro with 50 kV irradiation. This energy cannot be used on a living animal since most of the energy is absorbed before the irradiation reaches the dorsal part of the trachea. At this time our experiments comprise energies up to 4 MeV photons and electrons, different dose rates and different RBE-factors.

R.F. de Estable-Puig: Which are the features which allow you the identification in SEM of empty goblet cells?

Authors: In this laboratory the emptying of goblet cells from the small intestine has been worked out carefully (L. Friberg: Effects of irradiation on the small intestine of the rat. A SEM Study. Thesis Berlings, Arlöv, Sweden, 1980). There are similar findings in the actual system with small "sacs" with microvilli coming up to the surface as a result of the irradiation.

R.F. de Estable-Puig: In the first phase of the in vitro experiments, is the stimulation effect observed on all the ciliated surface or only in groups of cilia?

Authors: On all the ciliated surface.

R.F. de Estable-Puig: Which was the delay from the end of irradiation until irradiated and control sections were placed in the chamber, previous to the recording of the beatings?

Authors: 1 st rabbit 24 hrs. 2 nd rabbit 48 hrs after irradiation etc. The last rabbit was examined 240 hrs after.

H. Dalen: What is the explanation for the cellular mechanism leading to increased ciliary bleb formation after irradiation?

Authors: In fact we do not know. We see the same inside configuration of these blebs as you have published earlier. It is strange that irradiation can have this effect. We do not exclude other causes.

